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# Habitat Partitioning of Malaria Vectors in Nchelenge District, Zambia

Smita Das, Mbanga Muleba, Jennifer C. Stevenson, and Douglas E. Norris* for the Southern Africa International Centers of Excellence for Malaria Research Team

*W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland; Tropical Disease Research Centre, Ndola Central Hospital, Ndola, Zambia; Macha Research Trust, Choma, Zambia*

*Abstract.* Nchelenge District in Luapula Province, northern Zambia, experiences holoendemic malaria despite implementation of vector control programs. The major *Anopheles* vectors that contribute to *Plasmodium falciparum* transmission in this area had not previously been well defined. Three collections performed during the 2012 wet and dry seasons and the 2013 wet season revealed *Anopheles funestus* sensu stricto and *Anopheles gambiae* sensu stricto as the main vectors, where 80–85% of each collection was composed of *An. funestus*. Both vectors were found to be highly anthropophilic, and *An. funestus* has higher sporozoite infection rates (SIRs) and entomological inoculation rates (EIRs) year-round compared with *An. gambiae*: SIRs of 1.8–3.0% and 0–2.5%, respectively, and EIRs of 3.7–41.5 infectious bites per 6-month period (ib/p/6mo) and 0–5.9 ib/p/6mo, respectively. Spatial and temporal changes in each vector's dynamics and bionomics were also observed. *Anopheles funestus* was the predominant vector in the villages near Kenani Stream in both wet and dry seasons, whereas *An. gambiae* was found to be the main vector in areas near Lake Mweru during the wet season. The vector data illustrate the need for broader temporal and spatial sampling in Nchelenge and present unique opportunities to further our understanding of malarial transmission and implications for malarial control in high-risk areas.

## INTRODUCTION

*Anopheles funestus* sensu stricto and *Anopheles gambiae* sensu stricto are the most notorious vectors of *Plasmodium falciparum* malarial transmission in sub-Saharan Africa. Each of these species' distributions, their abilities to transmit the malarial parasite, and other demographic and environmental factors are heterogeneous and influence the local intensity of malarial transmission.[1] The relative composition of the vectors in areas where both *An. funestus* and *An. gambiae* are sympatric and their respective feeding behaviors vary from region to region in Africa. When both *An. funestus* and *An. gambiae* are present in a community, transmission can be exceedingly high.[1] This often occurs because these vector species exploit different breeding habitats and, although sympatric, may stagger their peak densities, which can prolong the transmission season.[1] *Anopheles gambiae* tends to oviposit in temporary breeding sites such as puddles and animal foot prints, which are abundant during the rainy season.[2–4] In contrast, *An. funestus* prefers more permanent breeding sites and populations tend to peak toward the end of the rainy season and into the first part of the dry season.[2–4] Both vectors have been shown to be highly anthropophilic, expressing a tendency to feed on humans, as well as being endophagic and endophilic, feeding and resting indoors, respectively.[2,4–7]

In communities where both vector species reside, there may be differences in the vector composition depending on the season and geography. From June 2003 to 2004 in western Kenya, it was found that *An. gambiae* s.s. was the major vector species driving transmission at one site in the lowlands and three sites in the highlands.[8] However, one of the villages in the lowland site had experienced recent larval habitat changes and *An. funestus* s.s. was found to be the major vector species.[8] In addition, there was pronounced spatial heterogeneity in vector composition and transmission rates in the lowland areas.[8,9] Differences in vector composition, biting behaviors, and transmission intensities among study sites that also vary temporally and spatially suggest that there is unequal risk of malarial transmission within an endemic area. Heterogeneity in malarial risk has implications for developing and targeting interventions because a single vector control measure such as indoor residual spraying (IRS) may successfully interrupt transmission in a low transmission setting, whereas multiple vector control measures such as IRS, long-lasting insecticide-treated nets (LLINs), larviciding, and/or larval habitat source reduction may be needed to have an impact in high transmission settings.[10–21] Therefore, it is necessary to identify and characterize malarial vectors at a local scale to inform successful control strategies.

Nchelenge District in Luapula Province, northern Zambia, experiences holoendemic transmission of *P. falciparum*.[22] Despite clinical evidence of high transmission rates, the mosquito vectors and their behaviors that influence transmission have not been well characterized. Preliminary mosquito collections by the southern Africa International Centers of Excellence for Malaria Research (ICEMR) in-country partner, the Tropical Disease Research Center (Ndola, Zambia), suggested that *An. funestus* s.s. and *An. gambiae* s.s. are the primary and secondary vectors, respectively, and together contribute to the maintenance of year-round *Plasmodium* transmission in Nchelenge (M. Muleba, unpublished data). The aims of this study were to confirm and characterize *An. funestus* s.s. and *An. gambiae* s.s., and their spatial and temporal patterns in two ecologically distinct habitats within Nchelenge District. This study provides fundamental and requisite information on vector dynamics of *An. funestus* s.s. and *An. gambiae* s.s. populations and their roles in malarial transmission in Nchelenge District.

## METHODS

**Study area.** This study was carried out in association with the Johns Hopkins Southern Africa ICEMR project. The focus

*Address correspondence to Douglas E. Norris, W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, 615 North Wolfe Street, Baltimore, MD 21205. E-mail: douglas.norris@jhu.edu

of the research reported here is Nchelenge District, Luapula Province, in northern Zambia (9°19′115″S, 28°45′070″E) at an elevation of approximately 807 m above sea level and in a marsh ecotype (Figures 1 and 2). The district lies along the eastern perimeter of Lake Mweru, which serves as the border between the southeastern part of the Democratic Republic of Congo and the northwestern part of Zambia. Flowing from south to north into Lake Mweru, Kenani Stream is also found in the area. There is a single rainy season from November to May, followed by a cool dry season from May to August and a hot dry season from August to November (Figure 3). Rainfall follows a seasonal pattern, with a peak of 2700 mm during the rainy months and close to 0 mm during the dry months (Figure 3). Longitudinal and cross-sectional households with prior enrollment in the ICEMR program and located within two defined 1-km$^2$ grids along both Lake Mweru (grid cell r34c5: 9°18′0.36″S, 28°44′25.08″E; gird cell r34c6: 9°18′2.16″S, 28°44′57.84″E) and Kenani Stream (grid cell r26c11: 9°22′19.74″S, 28°47′42.72″E; grid cell r29c10: 9°20′42.29″S, 28°47′9.6″E) were chosen for mosquito sampling. Mosquito collections were conducted at three villages (Katuna, Yenga, and Malulu) along Lake Mweru and two villages (Kapande B and Mutepuka) near Kenani Stream (Figure 2). These two collection areas are approximately 7 km apart and are representative of the local demography and landscape. People move between the two sites within the year as they switch from fishing to farming livelihoods and the two localities have high malarial incidence. Both LLINs and IRS with pyrethroids have been implemented in Nchelenge District since 2006 and 2007, respectively.

**Mosquito collection and handling.** Mosquitoes were collected by Center for Disease Control light trap (CDC LT) and pyrethroid spray catch (PSC) in the three lakeside villages (Katuna, Yenga, and Malulu) and two inland villages (Kapande B and Mutepuka) during the periods March 24–April 10, 2012 (wet), August 27–September 9, 2012 (dry), and March 5–April 25, 2013 (wet). Collection methods were approved by the Johns Hopkins Bloomberg School of Public Health Institutional Review Board (no. 00003467) and in Zambia (TDRC/ERC/2010/14/11).

CDC LTs and PSCs were set once per household in each sampling season, ensuring a 5-day gap between the two collection methods in the same household. In the wet 2012 collection, 11 lakeside and 11 streamside households were sampled, totaling 22 CDC LT and 22 PSC collections. During the 2012 dry collection, 21 lakeside and 15 streamside households were sampled using CDC LTs and PSCs, totaling 36 collections for each trapping method. In the 2013 wet collection, 39 lakeside and 38 streamside households were sampled, totaling 77 CDC LTs and 77 PSC collections.

CDC LTs were hung indoors next to sleeping persons, approximately 1 m above the floor, and would typically run from 6:00 PM to 6:00 AM. PSCs were performed in the morning (6:00 AM–10:00 AM). Briefly, white sheets were placed over all surfaces and a 100% synthetic aerosol pyrethroid was applied on the exterior eaves to prevent mosquito exit,


FIGURE 1. Nchelenge District field site is in northern Zambia and represents a site with unsuccessful malarial control.

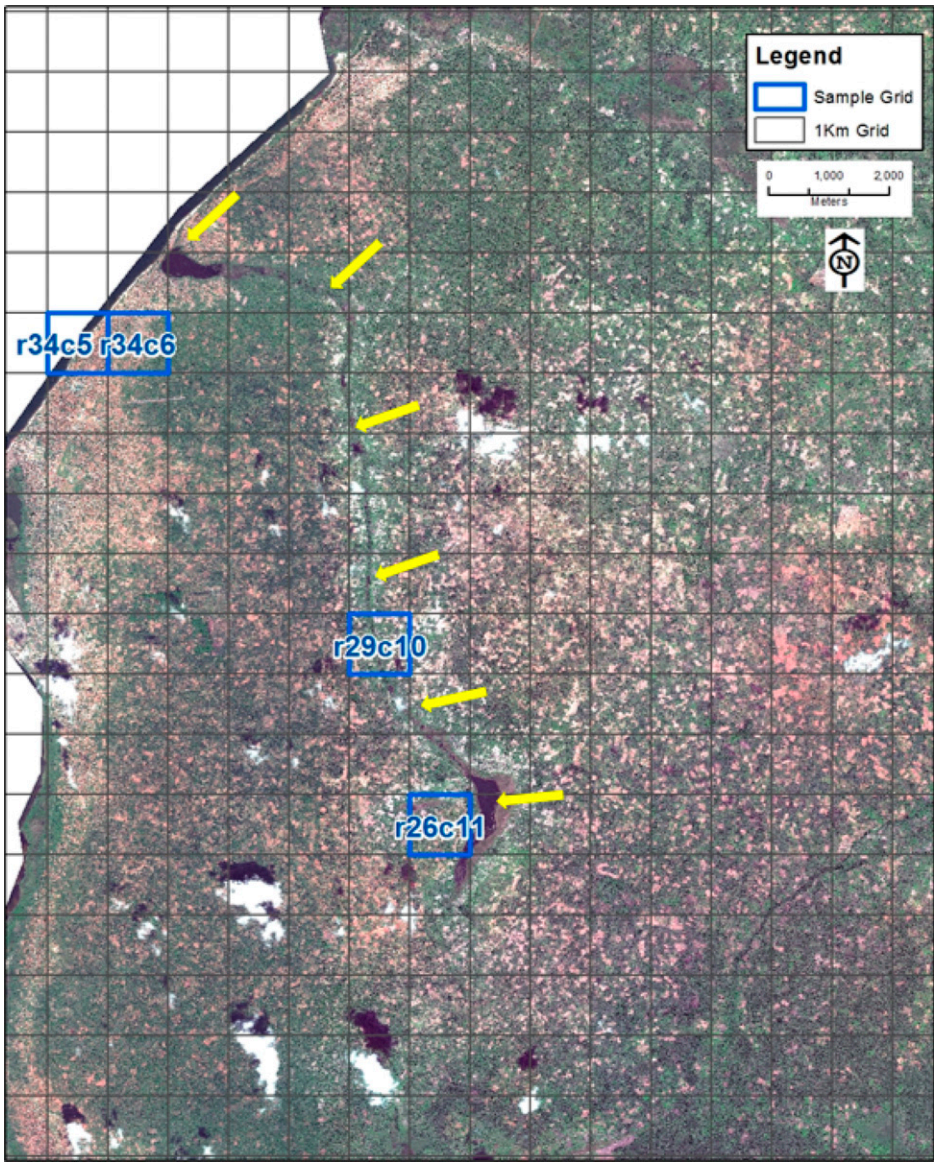


FIGURE 2. Satellite image of the study area in Nchelenge District. The 1-km$^2$ grids for study collections are highlighted in blue. Katuna, Yenga, and Malulu villages are located in grids r34c5 and r34c6, and Kapande B village is located in grid r29c10. The white area on the left side of the image represents Lake Mweru. The yellow arrows point to Kenani Stream that flows into Lake Mweru.

the ceiling, indoor eaves, and walls, and then the home closed. After approximately 15 minutes, the sheets were taken out of each household and knocked down mosquitoes were collected. All field-caught mosquitoes were killed by freezing. The female anopheline mosquitoes were then morphologically identified to species using a dichotomous key[3,23] and dissecting microscope (both vectors and nonvectors). Up to three mosquitoes were placed in each labeled 0.6 mL microcentrifuge tube containing silica gel desiccant and cotton wool, and stored either at room temperature or frozen at −20°C until laboratory processing, which took place at Johns Hopkins University Bloomberg School of Public Health in Baltimore, MD.

**DNA preparation and polymerase chain reaction (PCR).** For all collected anophelines, the head and thoraces were separated from the abdomen, and DNA extraction of the abdomen was performed using a modified salt extraction.[24] The morphological identification of anopheline mosquitoes was confirmed using a PCR specific for members of the *An. gambiae* or *An. funestus* species complexes as described previously.[25–28]

If a mosquito sample could not be molecularly confirmed as either *An. gambiae* or *An. funestus*, then an internal transcribed spacer 2 (ITS2) PCR was used.[26] The ITS2 PCR is robust and has a range of base pair sizes that are specific to other anopheline species common to sub-Saharan Africa.[26] Briefly, the forward primer was ITS2A: 5′-TGT GAA CTG CAG GAC ACA T-3′ and the reverse primer was ITS2B: 5′-TAT GCT TAA ATT CAG GGG GT-3′[26] The 25 μL reaction mixture contained 2.5 μL 10× reaction buffer, 1 mM $MgCl_2$, 2.5 mM dNTPs, 30 pmol of each primer, 2.0 units Taq polymerase, 1 μL extracted DNA, and sterile water.[26]

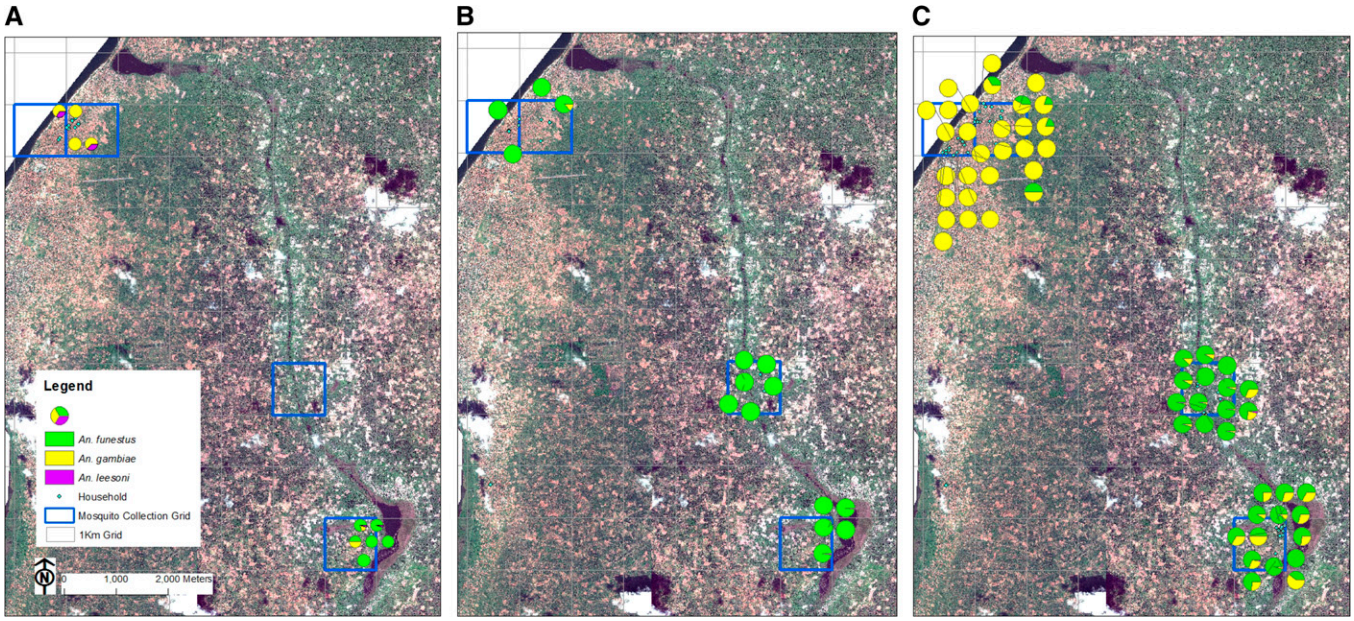


FIGURE 3. The proportion of *Anopheles* species caught per household that were sampled during the (**A**) March 24–April 10, 2012 (wet) collection, (**B**) August 27–September 9, 2012 (dry) collection, and (**C**) the March 5–April 25, 2013 (wet) collection. Species are denoted by color: *Anopheles funestus* sensu stricto (green), *Anopheles gambiae* sensu stricto (yellow), and *Anopheles leesoni* (purple).

The PCR was performed with an initial denaturation for 2 minutes at 94°C, followed by 40 cycles at 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 40 seconds, and then a final extension step was 10 minutes at 72°C.[26]

The mosquitoes that were molecularly confirmed as *An. gambiae* s.s. were also tested for molecular M-form (now recognized as *Anopheles coluzzii*) or S-form as detailed by Favia and others.[29] Briefly, the 5′ end of the intergenic recombinant DNA spacer region is amplified to produce a 727 base pair band for M-form, 475 base pair band for S-form, and both bands for hybrid specimens.[29]

All specimens, regardless of being visually fed or unfed, were then tested for host animal blood using the Kent and Norris[24] multiplex PCR, which amplifies the cytochrome b gene of the mitochondrial genome producing a range of mammalian host-specific bands from 132 to 680 base pairs.[30] A modification to this PCR was made to better detect a human host blood meal; the original forward primer to detect a human blood meal was removed and replaced with a new forward and reverse primer set that amplifies a human-specific 193 base pair region of the cytochrome B gene. The forward primer was FOR16068: 5′-GAC TCA CCC ATC AAC AAC CG-3′, and the reverse primer was REV16260: 5′-GGC TTT GGA GTT GCA GTT GA-3′. Amplification conditions were not altered for this primer replacement.

Samples that did not produce a band(s) for host blood meal with the modified multiplex PCR were then screened with a more sensitive PCR, which amplifies a conserved 98 base pair region of the cytochrome b gene of the mitochondrial genome of the mammalian host, followed by a restriction fragment length polymorphism assay to identify animal host of interest.[31] The identification of the mammalian host was used to calculate the human blood index (HBI), the proportion of human blood meals relative to the total blood meals, for each vector species. For mixed blood meals, they were counted as a blood meal for each host animal.

The abdominal DNA for each anopheline was then tested for the presence of the *P. falciparum* parasite by PCR.[32] This PCR amplifies a small portion of the cytochrome b gene of *P. falciparum* and is more sensitive than the commonly used Snounou and others[32] PCR, producing a fragment with an expected size of 183 base pairs.[33]

**Circumsporozoite protein enzyme-linked immunosorbent assay (CSP-ELISA) for *P. falciparum* detection.** The CSP-ELISA method as described by the Malaria Research and Reference Reagent Resource Center (MR4) was used to detect *P. falciparum* CSP in the mosquito head and thorax. The values associated with each mosquito that are two times the mean of the negative controls on the plate were considered to be *P. falciparum* positive. CSP-ELISA was performed on all *Anopheles* mosquitoes collected in the wet and dry seasons of 2012. However, due to the large numbers of anophelines caught during the March–April 2013 collection, a subsample of mosquitoes was selected for CSP-ELISA. The subsample was representative of all collection dates and also provided a large enough sample size for each species for statistical analyses: 695/2,417 (28.8%) *An. funestus* s.s. and 520/564 (92.2%) *An. gambiae* s.s.

**Spatial and temporal mapping of malarial vectors.** To map the presence of malarial vectors and their proportions within each household in Nchelenge District both spatially and temporally, ArcMap 2.0 (ESRI ArcGIS) software was used to create a layer of vector composition and density per households over a satellite image of Nchelenge District. This was done for each collection period to visualize changes in vector composition and abundances spatially and temporally.

**Human biting rate (HBR) and entomological inoculation rate (EIR).** The average number of bites per person per

night (HBR) was calculated by dividing the number of PCR-confirmed *An. funestus* s.s. and *An. gambiae* s.s. vectors from CDC LTs by the number of people sleeping in the household the night of the collection. Because CDC LT collections have been shown previously in western Kenya and Zambia to be a reasonable correlate for estimating the HBR when human landing catches cannot be performed, EIR calculations from CDC LT collections were regarded as the best measurement of transmission intensity.[34–37]

The seasonal EIR for each vector species per household was calculated as the product of the HBR, sporozoite infection rate (SIR), and 180 days (6 months for each season), as collections were conducted in either the wet or dry seasons. The SIR is defined as the proportion of infectious vectors in a household or total collection. The overall EIR estimated for Nchelenge District was the average of all household EIRs. For the March–April 2013 collection where the sample size was sufficiently large, overall EIR calculations were further partitioned among lakeside and streamside villages.

**Statistical analysis.** Abundances, SIR, and EIR of *An. funestus* s.s. and *An. gambiae* s.s. in the March–April 2012 (wet), August–September 2012 (dry), and March–April 2013 (wet) collections were compared using STATA version 11 (Stata Corp., College Station, TX). For comparison of vector abundances and EIRs, the negative binomial regression model for overdispersed data was used. Logistic regression model was used to compare SIRs. A $P$ value $< 0.05$ was considered statistically significant. The kappa statistic was calculated to classify the agreement between CSP-ELISA and PCR method results as "poor" (< 0.40), "fair" (0.40–0.70), and "excellent" (> 0.70).

## RESULTS

**Seasonal variation.** During the 2012 wet season, a total of 366 *Anopheles* mosquitoes were collected using CDC LTs and PSCs and were composed of 83.3% ($N = 305$) *An. funestus* s.s. (henceforth referred to as *An. funestus*), 9.0% ($N = 33$) *An. gambiae* s.s. (henceforth referred to as *An. gambiae*), and 7.7% ($N = 28$) *An. leesoni* (Figure 4). Within the collection, the abundance of *An. funestus* was greater than *An. gambiae* per household per CDC LT night (relative risk [RR] = 5.6, 95% confidence interval [CI] = 2.2, 14.3, $P > 0.001$) and per PSC trap morning (RR = 19, 95% CI = 6.8, 53.2, $P < 0.001$).

In the dry season of 2012, collections from CDC LTs and PSCs were composed of 1,324 anophelines, of which 99.3% ($N = 1,315$) were *An. funestus*, 0.6% ($N = 8$) were *An. gambiae*, and 0.1% ($N = 1$) were *An. leesoni*. The abundance of *An. funestus* caught per household was higher than *An. gambiae* in both CDC LT collections per trap night (RR = 130, 95% CI = 42, 404, $P < 0.001$) and PSC collections per trap morning (RR = 253.2, 95% CI = 54.1, 1187.2, $P < 0.001$).

A total of 2,989 *Anopheles* mosquitoes were caught in the 2013 wet season using CDC LTs and PSCs. The majority of the collection was made up of *An. funestus* (80.9%, $N = 2,417$), followed by *An. gambiae* (18.9%, $N = 564$) and *An. leesoni* (0.2%, $N = 8$). Within the collection, the number of collected *An. funestus* per household was greater than *An. gambiae* per CDC LT per night (RR = 3.9, 95% CI = 2.5, 5.9, $P > 0.001$) and PSCs per trap morning (RR = 5.1, 95% CI = 3.0, 8.8, $P < 0.001$).

When comparing individual species abundance among collection seasons, the 2012 and 2013 wet collections had smaller catches of *An. funestus* per household per CDC LT trap night (2012 wet collection: RR = 0.14, 95% CI = 0.04, 0.50, $P < 0.004$; 2013 wet collection: RR = 0.25, 95% CI = 0.04, 0.52, $P = 0.015$) and PSC trap morning (2012 wet collection: RR = 0.26, 95% CI = 0.11, 0.63, $P = 0.003$; 2012 dry collection: RR = 0.55, 95% CI = 0.26, 1.17, $P = 0.121$) when compared with the 2012 dry season collection. In contrast, both the 2012 and 2013 wet season collections had larger abundances of *An. gambiae* per household per CDC LT night (2012 wet collection: RR = 3.5, 95% CI = 1.1, 11.1, $P = 0.04$; 2013 wet collection: RR = 9.2, 95% CI = 3.4, 24.8, $P < 0.001$)

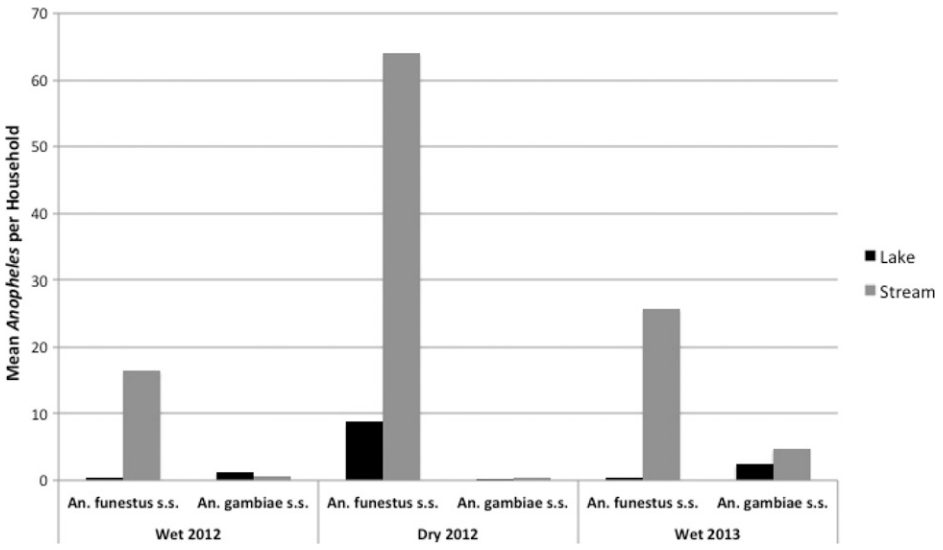


FIGURE 4. Mean number of *Anopheles funestus* sensu stricto and *Anopheles gambiae* sensu stricto collected using Center for Disease Control light trap and pyrethroid spray catch collection methods in lakeside and streamside households in the 2012 wet, 2012 dry, and 2013 wet collection periods.

or PSC trap morning (2012 wet collection: RR = 3.5, 95% CI = 0.64, 19.1, $P = 0.15$; 2013 wet collection: RR = 27, 95% CI = 5.8, 125.9, $P < 0.001$) than the 2012 dry season collection.

The majority of the collected *An. gambiae* were successfully identified as S-form (527/564; 93.4%). A small proportion of the collected *An. gambiae* (37/564; 6.6%) could not be identified to molecular form due to failed PCRs that were attempted twice.

**Spatial variation.** In the wet season of 2013, spatial differences in vector composition were evident when comparing collections from villages near Lake Mweru to those located inland along Kenani Stream. In the lakeside villages (Katuna, Yenga, and Malulu), 86 collections from 39 households resulted in a total of 133 anophelines caught, of which the majority was *An. gambiae* (85.7%, $N = 114$), with the remainder *An. funestus* (14.3%, $N = 19$). In 99 collections from 38 households in the streamside villages (Kapande B, Mutepuka), 2,847 anophelines were collected and were composed of 2,397 *An. funestus* (84.2%) and 450 *An. gambiae* (15.8%). The streamside collection revealed higher numbers of both *An. funestus* and *An. gambiae* caught per household per CDC trap night than the lakeside collection (*An. funestus*: RR = 42.7, 95% CI = 21.8, 83.7, $P < 0.001$; *An. gambiae*: RR = 1.7, 95% CI = 1.1, 2.8, $P = 0.03$). Similarly, greater numbers of *An. gambiae* were caught per household per PSC trap morning in streamside villages compared with lakeside villages (RR = 2.4, 95% CI = 0.97, 6.1, $P = 0.05$). No *An. funestus* were caught in streamside households by PSC traps.

When species composition was studied within a locality during the wet season of 2013, there were significant differences in the abundance of *An. funestus* and *An. gambiae* caught both within the lakeside and the streamside areas. In the lakeside collection, more *An. gambiae* were caught than *An. funestus* (RR = 4.8, 95% CI = 2.5, 9.3, $P < 0.001$) per household per CDC LT night, whereas in the streamside households, there were more *An. funestus* than *An. gambiae* per CDC LT night (RR = 5.3, 95% CI = 3.9, 7.3, $P < 0.001$). For PSC collections per trap morning, no *An. funestus* were caught lakeside. In contrast, near the stream, *An. funestus* was the dominant vector compared with *An. gambiae* per PSC trap morning (RR = 5.1, 95% CI = 3.4, 7.7, $P < 0.001$).

**Blood-feeding behavior.** Mammalian host was assessed for all anophelines due to the possibility that there may be mosquitoes that appear "unfed," but are actually fed as determined by molecular assays.[24,31,38] HBI was, therefore, calculated using PCR-confirmed blood meals.

In the 2012 wet collection, 111/343 *An. funestus* (32.4%), 9/36 *An. gambiae* (25%), and 6/32 *An. leesoni* (18.8%) were visually fed. All blood meals for *An. funestus* and *An. gambiae* were identified as human, whereas five of the *An. leesoni* fed on humans and one *An. leesoni* had fed on goat. Of those anophelines that were visually "unfed," five were found to have human blood meals: 2/232 *An. funestus* (0.9%), 1/27 *An. gambiae*, (3.7%), and 2/26 *An. leesoni* (7.7%). The HBI for both *An. funestus* and *An. gambiae* was 1.0 and 0.88 for *An. leesoni*.

In the dry season of 2012, 215/1,315 *An. funestus* (16.5%), 2/8 *An. gambiae* (25%), and 0/1 *An. leesoni* (0%) were visually fed. With the exception of two *An. funestus* blood meals identified as goat, all fed mosquitoes had taken human blood meals. Of those anophelines that were visually "unfed," 45/1,098 (4.1%) were identified as *An. funestus* and were human fed. The HBIs for *An. funestus* and *An. gambiae* were 0.99 and 1.0, respectively.

In the wet season collection of 2013, 444/2,417 (18.4%) *An. funestus* and 100/564 (17.7%) *An. gambiae* were visually fed. No visually fed *An. leesoni* ($N = 8$) were observed. Of those visually fed, 420 *An. funestus* and 94 *An. gambiae* specimens had taken blood meals exclusively from human hosts. Mixed human and goat blood meals were detected in 24 *An. funestus* and six *An. gambiae*. One *An. funestus* mosquito fed on goat exclusively. Of the anophelines that were classified as "unfed," 164/2,445 (6.7%) were found to contain mammalian blood by PCR; 153 *An. funestus* and 11 *An. gambiae*. One hundred and forty-six of the 153 *An. funestus* (95.4%) and 8/11 (72.7%) *An. gambiae* with blood meals had fed on human hosts. Seven blood meals taken by *An. funestus* (7/153) and three blood meals taken by *An. gambiae* (3/11) were mixed blood meals of human and goat. The HBIs for *An. funestus* and *An. gambiae* were 0.95 and 0.93, respectively.

**CSP-ELISA versus PCR detection of *P. falciparum*.** The CSP-ELISA method of *P. falciparum* detection for both *Anopheles* vectors was compared with the PCR method by calculating the kappa value for each collection. It was observed that most *Anopheles* specimens that were positive by CSP-ELISA were not positive by PCR, and vice versa. As a result, the kappa values for the March–April 2012 (wet), August–September 2012 (dry), and March–April 2013 (wet) collections were 0.0, 0.23 (standard error [SE] = 0.073, 95% CI = 0.092, 0.380), and 0.20 (SE = 0.067, 95% CI = 0.071, 0.334), respectively, suggesting poor agreement between the two methods for all field collections.

**Comparison of SIR and EIR of vectors within collections.** In the 2012 wet collection, *An. funestus* had an SIR of 1.8% by CSP-ELISA and a seasonal EIR of 3.7 infectious bites per 6-month period (ib/p/6mo) (Table 1). No infectious *An. gambiae* by CSP-ELISA were detected. The 2012 dry collection revealed that *An. funestus* had an SIR of 2.4% and an EIR of 41.5 ib/p/6mo (Table 1). During the 2012 dry collection, CDC LTs caught only six *An. gambiae* and none were positive for *P. falciparum*. Because no infectious *An. gambiae* were caught in the wet and dry collections of 2012, logistic regression comparing *An. gambiae* and *An. funestus* SIRs could not be performed. There was no statistically significant difference in both vector's EIRs within the 2012 wet and 2012 dry collections (2012 wet: RR = 18.3, 95% CI = −3585.8, 3622.3, $P = 0.99$; 2012 dry: RR =19, 95% CI = −1999.4,

TABLE 1

Total abundance, SIR, and EIR for *Anopheles funestus* sensu stricto and *Anopheles gambiae* sensu stricto based on CDC LT collections during the 2012 wet, 2012 dry, and 2013 wet seasons in Nchelenge District

| Collection | *Anopheles* spp. | SIR (%) | EIR (ib/p/6mo) |
|---|---|---|---|
| Wet 2012 ($N = 158$) | *An. funestus* s.s. ($n = 134$) | 1.8 | 3.7 |
| | *An. gambiae* s.s. ($n = 24$) | 0.0 | 0.0 |
| Dry 2012 ($N = 787$) | *An. funestus* s.s. ($n = 781$) | 2.4 | 41.5 |
| | *An. gambiae* s.s. ($n = 6$) | 0.0 | 0.0 |
| Wet 2013 ($N = 856$) | *An. funestus* s.s. ($n = 480$) | 3.0 | 39.6 |
| | *An. gambiae* s.s. ($n = 376$) | 2.5 | 5.9 |

CDC LT = Center for Disease Control light trap; EIR = entomological inoculation rate; ib/p/6mo = infectious bites per 6-month period; SIR = sporozoite infection rate.

TABLE 2

Total abundance, SIR, and EIR for *Anopheles funestus* sensu stricto and *Anopheles gambiae* sensu stricto from CDC LT collections in the lakeside villages (r34c5, r34c6; Katuna, Yenga, and Malulu) and streamside villages (r29c10, r26c11; Kapande B and Mutepuka) during the 2013 wet season in Nchelenge District

| Wet 2013 | *Anopheles* spp. | SIR (%) | EIR (ib/p/6mo) |
|---|---|---|---|
| Lakeside | *An. funestus* s.s. ($N$ = 19) | 0.0 | 0.0 |
| | *An. gambiae* s.s. ($N$ = 98) | 1.5 | 0.60 |
| Streamside | *An. funestus* s.s. ($N$ = 461) | 3.9 | 51.5 |
| | *An. gambiae* s.s. ($N$ = 278) | 3.4 | 10.1 |

CDC LT = Center for Disease Control light trap; EIR = entomological inoculation rate; ib/p/6mo = infectious bites per 6-month period; SIR = sporozoite infection rate.

2037.4, $P$ = 0.99). In the wet season collection of 2013, both *An. funestus* and *An. gambiae* had SIRs of 3.0% and 2.5% (Table 1) and the EIRs were 39.6 ib/p/6mo and 5.9 ib/p/6mo for *An. gambiae*, respectively. There were no significant differences in SIRs ($\chi^2$ = 3.44, 95% CI = 0.07, 2.2, $P$ = 0.065) and EIRs (RR = 0.11, 95% CI = −3.2, 3.4, $P$ = 0.95) between *An. funestus* and *An. gambiae*.

During the 2013 wet collection, spatial variation in vector EIRs and SIRs was studied between the Lake Mweru and Kenani Stream villages (Table 2). Along the lakeside, *P. falciparum* was detected by CSP-ELISA in only *An. gambiae*, resulting in an SIR of 1.5% and an EIR of 0.60 ib/p/6mo (Table 2). In contrast, both *An. funestus* and *An. gambiae* near the stream were found to be infectious. Higher infectivity rates were detected than the anopheline vectors found along the lakeside: SIRs of 3.9% and 3.4%, respectively (Table 2); however, the lack of infectious *An. funestus* in the lakeside villages prohibited logistic regression analysis to statistically compare the SIRs between the two localities. For *An. gambiae*, the SIRs between the villages near the lake and stream were not significantly different ($\chi^2$ = 3.97, 95% CI = −0.34, 3.9, $P$ = 0.10). The seasonal EIRs in the streamside collections were also higher than the lakeside collections for *An. funestus* and *An. gambiae*, 51.2 ib/p/6mo and 10.1 ib/p/6mo, respectively, but again this difference was not statistically significant for either *An. funestus* (RR = 20.1, 95% CI = −2027.7, 2067.9, $P$ = 0.99) or *An. gambiae* (RR = 2.9, 95% CI = −0.10, 5.9, $P$ = 0.06).

Spatial differences in SIRs and EIRs of vectors were also investigated within the lakeside and streamside areas in the 2013 wet collection. Within the lakeside collection, there was no statistical significance between the *An. funestus* and *An. gambiae* EIRs (RR = 18.9, 95% CI = −10930.4, 10968.3, $P$ = 0.997). No infectious *An. funestus* were caught near the lake. As a result, logistic regression could not be performed to compare its SIR with that of *An. gambiae*. In the streamside collections, there was a significantly higher proportion of infectious *An. funestus* per trap night than *An. gambiae* ($\chi^2$ = 5.79, 95% CI = 0.19, 2.10, $P$ = 0.019), although there was no statistically significant difference in EIRs between *An. funestus* and *An. gambiae* in the streamside villages (RR = 1.61, 95% CI = −0.29, 3.52, $P$ = 0.096).

**Comparison of SIR and EIR between collections.** The SIRs and EIRs for each vector were compared among the 2012 and 2013 collections. There was a difference in both the proportion of infectious *An. funestus* in the 2012 wet collection compared with 2012 dry collection ($\chi^2$ = 5.19, 95% CI = 0.17, 3.5, $P$ = 0.031) and the EIR (RR = 2.4, 95% CI = 0.41, 4.4, $P$ = 0.019). A difference in the proportion of infectious *An. funestus* between the 2013 wet collection and the 2012 dry collection ($\chi^2$ = 7.29, 95% CI = 0.46, 3.2, $P$ = 0.009) was also observed, but there was no significant difference in the EIRs (RR = 0.05, 95% CI = −2.5, 2.6, $P$ = 0.971). There was no statistically significant difference in the SIRs and EIRs of *An. funestus* between the 2012 and 2013 wet collections (SIR: $\chi^2$ = 0.0, 95% CI = −1.5, 1.5, $P$ = 0.967; EIR: RR = 2.4, 95% CI = −0.69, 5.4, $P$ = 0.129). No infectious *An. gambiae* were caught in the 2012 wet and dry collections. As a result, logistic regression and negative binomial regression comparing *An. gambiae* SIRs and EIRs, respectively, between collection periods could not be performed.

## DISCUSSION

In this study, malarial vector transmission dynamics were characterized in two distinct ecologies in Nchelenge District, Zambia, over three consecutive seasons: 2012 wet, 2012 dry, and 2013 wet. The densities of *An. funestus* and *An. gambiae* varied markedly between wet and dry seasons and all collections revealed microspatial dynamics of *An. funestus* and *An. gambiae* in villages located along Lake Mweru and Kenani Stream. During the wet seasons, *An. gambiae* was the primary vector found in lakeside villages, whereas *An. funestus* and *An. gambiae* were the primary and secondary vectors, respectively, in interior streamside villages. The dry season collection suggested that *An. funestus* is the dominant vector of transmission in both the lakeside and streamside villages during periods of low rainfall, when only a few sympatric *An. gambiae* were caught. It should also be noted that lakeside mosquito densities were a fraction of inland and streamside densities, regardless of season (Figure 4). The abundances of *An. gambiae* in both the 2012 and 2013 wet collections were significantly greater than the 2012 dry collection. This is consistent with previous reports of *An. gambiae* proliferation at the beginning of the wet season, whereas *An. funestus* increases toward the end of the wet season and dominates in the dry season.[39,40] The increases in *An. gambiae* mosquitoes are likely due to a wide range of temporary sunlit breeding habitats found throughout Nchelenge District such as animal footprints, puddles, and ground depressions formed due to heavy rains during the wet season.[3,41,42] In villages near Lake Mweru, *An. gambiae* larvae and pupae in oviposition surveys have been collected in puddles and boats onshore along the lake, as well as in ditches along the main road (unpublished data). In contrast, *An. funestus* typically prefer vegetated, semipermanent to permanent breeding sites such as swamps and marshes, which are abundant in Nchelenge, but are likely to be washed out during heavy rain.[3,41,43] Overall, *An. funestus* was found throughout all collection seasons at high densities, especially inland near Kenani Stream, which likely serves as a stable habitat with permanent marsh and swamps along the stream channel that remain flooded through the dry season.

Both *An. funestus* and *An. gambiae* were found to be highly anthropophilic, but surprisingly a few mixed human/goat and goat only blood meals were identified in both vectors, which has been infrequently reported.[40,44–46] In Nchelenge District, goats are occasionally kept indoors at night and may serve as an incidental host to indoor foraging *An. funestus* and

*An. gambiae*. *Anopheles funestus* and *An. gambiae* are widely known for their anthropophilic, as well as endophilic and endophagic behavior,[18,47,48] so it is not unexpected that in Nchelenge District, both vectors have a tendency to feed almost exclusively on humans.[3] During the study it was observed that inhabitants often participate in social gatherings into the late evening, indoors and outdoors. As a result, we cannot overlook the possibility of indoor and/or outdoor exposure to these two vectors prior to being under a bed net that may also contribute to malarial transmission.[49–54]

Each vector's role in transmission was further defined by determining the SIR for both *An. funestus* and *An. gambiae*. The agreement between SIRs determined by CSP-ELISA and PCR methods for each collection was considered "poor" for all collections. There have been several studies that have reported false CSP-ELISA positive results compared with microscopy and PCR.[55–59] Other studies have been unable to identify the cross-reacting antigens suspected of leading to false CSP-ELISA results, but have shown that the unknown antigen is heat unstable and can be removed by heating the CSP-ELISA homogenate to 100°C for 10 minutes.[55,60] Because this method of heating CSP-ELISA homogenate was not performed for this study, it is essential that future research confirm initial CSP-ELISA results with a second-heated CSP-ELISA or confirming infection with another diagnostic method. The secondary method used in this study, detecting *Plasmodium* spp. in mosquitoes by PCR can detect as little as 10 sporozoites, whereas CSP-ELISA requires at least 100 sporozoites for detection.[55] The PCR performed in this study detects all stages of *Plasmodium* spp., and due to inefficient recovery of DNA from the ELISA homogenate of mosquito head and thorax was carried out on the mosquito abdomen only. A positive result by PCR from the abdomen, therefore, does not necessarily imply salivary gland infection and could lead to overestimation of the SIR.[55] CSP-ELISA is often the preferred method as it detects only the human-infectious stage of the parasite. However, a sample that is positive by CSP-ELISA and by PCR of the abdomen would strongly indicate infection with *P. falciparum.* In this study, only 15 of 99 *An. funestus* and 0 of 26 *An. gambiae* were positive by both methods (Table 3), suggesting that there is very little agreement between CSP-ELISA and PCR.

The SIRs and subsequent EIRs reported in this study are based on the CSP-ELISA method, which is more specific to salivary gland infection compared with PCR and more widely used for EIR estimations,[55,61–63] allowing for comparison with other studies. The *An. funestus* SIR and EIR in the 2012 dry collection was higher than the 2012 wet collection. The large proportion of infectious *An. funestus* found within households during the dry season are likely due to stable breeding habitats and low rainfall, factors that support survival of the *An. funestus* population.[3,64–71] The longevity of *An. funestus* is particularly important because a higher proportion of malarial parasites can successfully develop and, combined with frequent exposure to infections still present in the local human population, can continue transmission into the wet season. However, when comparing the SIR and EIRs of *An. funestus* from the 2012 dry and 2013 wet periods, the wet collection parameters were higher than the dry collection suggesting that a larger sampling, especially during the dry season, would help better define the seasonal transmission dynamics. A comparison of the wet collections suggested a higher *An. funestus* EIR in 2013 than 2012. During the 2013 wet collection, only infected *An. gambiae* were found in the lakeside relative to *An. funestus*, and a higher, but not significant, SIR was observed in streamside *An. gambiae*. Due to the low number of infectious specimens, sample size may have limited the ability to detect significance, particularly along Lake Mweru, where low densities of mosquitoes reside compared with areas inland and near Kenani Stream. In the streamside villages, the *An. funestus* SIR was greater than that of *An. gambiae*. The large numbers of both *An. funestus* and *An. gambiae* in the inland streamside collections helped define patterns in SIRs and EIRs, whereas differences in numbers of the two species in the relatively small lakeside collection were not significant. The trend suggests that *An. gambiae* is the predominant vector lakeside. In the streamside villages, the *An. funestus* EIR was higher than that for *An. gambiae*.

General trends for *An. funestus* and *An. gambiae* EIRs across the collection periods show that transmission by *An. funestus* is highest during the dry season when *An. gambiae* has either low abundance or is absent. Throughout the wet seasons, *An. funestus* remains the dominant vector of transmission, but at a lower intensity than in the dry season, likely due to larval habitat washout during the rains. *Anopheles gambiae* contributes very little to transmission during the dry season and then serves as a secondary vector with an EIR similar to that of *An. funestus* in the wet season. As a result, malarial transmission in Nchelenge District is maintained year-round primarily by *An. funestus*. Spatially, transmission intensity of malaria is much higher in the inland streamside villages with high EIRs by both *An. funestus* and *An. gambiae*. In the lakeside villages, *An. gambiae* appears to be the dominant malarial vector, as no infectious *An. funestus* were detected in this study, likely an artifact of the small sample size. As Nchelenge endures a high burden of malaria year-round (J. Pinchoff, unpublished data), it is likely that these two vectors drive transmission across the district. Here, both vectors are highly resistant to pyrethroids (M. Muleba,

TABLE 3

Comparison of *Anopheles funestus* sensu stricto and *Anopheles gambiae* sensu stricto sporozoite infection rates by CSP-ELISA, PCR, and both CSP-ELISA and PCR combined for the 2012 wet, 2012 dry, and 2013 wet collections

| | *Anopheles* spp. | CSP-ELISA | PCR | CSP-ELISA and PCR |
|---|---|---|---|---|
| Wet 2012 | *An. funestus* s.s. ($N$ = 343) | 8 (2.3%) | 0 (0%) | 0 (0%) |
| | *An. gambiae* s.s. ($N$ = 36) | 0 (0%) | 0 (0%) | 0 (0%) |
| Dry 2012 | *An. funestus* s.s. ($N$ = 1315) | 31 (2.4%) | 32 (2.4%) | 8 (0.61%) |
| | *An. gambiae* s.s. ($N$ = 8) | 0 (0%) | 0 (0%) | 0 (0%) |
| Wet 2013 | *An. funestus* s.s. ($N$ = 676) | 19 (2.8%) | 24 (3.6%) | 7 (1%) |
| | *An. gambiae* s.s. ($N$ = 505) | 16 (3.2%) | 10 (1.98%) | 0 (0%) |

CSP-ELISA = circumsporozoite enzyme-linked immunosorbent assay; PCR = polymerase chain reaction.

unpublished data), which may explain why previous IRS campaigns have shown little effect in controlling malaria in Nchelenge.[59]

The present study demonstrates that *An. funestus* and *An. gambiae* are the major vectors of malarial transmission in Nchelenge District, Zambia. During the wet season, both vectors contribute to *P. falciparum* transmission, whereas during the dry season, only *An. funestus* appears to maintain transmission. Moreover, the villages found inland along Kenani Stream endure much higher biting rates of *An. funestus* and *An. gambiae* than the areas along Lake Mweru, where *An. gambiae* appears to be the primary vector during the rainy season. It is especially alarming that despite significant spatial differences in vector composition and transmission intensity at these two representative sites in Nchelenge, high year-round transmission in maintained across these habitats. The characterization of major *Anopheles* vectors in Nchelenge in three consecutive seasons and across habitats will further guide development and implementation of effective vector control strategies in this area.

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Authors' addresses: Smita Das and Douglas E. Norris, W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, E-mails: smita.das988@gmail.com and douglas.norris@jhu.edu. Mbanga Muleba, Tropical Disease Research Centre, Ndola Central Hospital, Ndola, Zambia, E-mail: MulebaM@tdrc.org.zm. Jennifer C. Stevenson, W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, and Macha Research Trust, Choma, Zambia, E-mail: jennyc.stevenson@macharesearch.org.

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