Changes in Anopheles funestus Biting Behavior Following Universal Coverage of Long-Lasting Insecticidal Nets in Benin.

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Running Title:

Behavioural adaptation of malaria vector to LLIN

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Authors declare that they have no competing interests.

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Abstract (200 words)

Background

Behavioural modification of malaria vectors in response to vector control methods is of great concern. We investigated whether full coverage of Long-Lasting Insecticide-treated mosquito Nets (LLIN) may induce a switch in biting behaviour in *Anopheles funestus*, a major malaria vector in Africa.

Method

Human-landing collections were conducted indoor and outdoor in two villages (Lokohouè and Tokoli) in Benin prior, 1 year and 3 years after implementation of universal LLIN coverage. Proportion of Outdoor Biting (POB) and Median Catching Times (MCT) were compared. The resistance of *An. funestus* to deltamethrin was monitored using bioassays.

Findings

MCT of *An. funestus* switched from 02:00 in Lokohoué and 03:00 in Tokoli to 05:00 after 3 years (Mann-Whitney p-value<0.0001). In Tokoli, POB increased from 45% to 68.1% (OR=2.55;95CI=1.72-3.78;p<0.0001) 1 year after the universal coverage whereas POB was unchanged in Lokohoué. In Lokohoué, however, the proportion of *An. funestus* that bites after 06:00 was 26%. Bioassays showed no resistance to deltamethrin.

Conclusion

This study provides evidence for a switch in malaria vectors biting behaviour following the implementation of LLIN at universal coverage. These findings might have direct consequences for malaria control in Africa and highlighted the need for alternative strategies for better targeting malaria vectors.

Keywords

Malaria; Vector control; Insecticide-Treated mosquito nets; Biting behaviour; Anopheles; Africa;

Text (3077 words)

Background

During the last decade, mortality and prevalence of malaria decreased substantially in sub-Saharan Africa [1]. Relying on increased international funding and massive implementation of vector control strategies, malaria elimination is back on the global health agenda [2]. Unfortunately, recent evidences of malaria resurgence have been recorded in several countries underlying limitations in the efficacy of the Long Lasting Insecticidal Nets (LLINs) and Indoor Residual Sprayings of insecticide (IRSs) [1, 3-5].

These vector control strategies are based on early characterization of the behavioural ecology of the main malaria vectors in Africa, *An. gambiae* and *An. funestus* [6]. Both interventions target vectors when they feed and/or rest indoors [7]. However, as describe by Fergusson et al. [8], there are many ecological reasons for all vectors to not be targeted by an insecticide e.g. insecticide resistance, behavioural avoidance, vector biodiversity, etc… Implication of pyrethroid resistance in the reduction of LLIN effectiveness [3, 9-11] was recently reported in West Africa although no clear evidence for an operational vector control failure could be yet demonstrated. Renewed interest recently emerged regarding the behavioural changes of mosquitoes following the implementation of vector control interventions [12]. Indeed, recent evidences suggested that malaria vectors may avoid the contact with the insecticide by either feeding predominantly outdoor or in the early evening [12-14]. This behavioural modulation

may result from the selection of genetically inherited traits or from phenotypic plasticity in response to increased coverage of LLINs and/or indoor residual sprayings. Moreover, Lefèvre *et al.* recently showed phenotypic plasticity in blood-feeding behaviour in *Anopheles gambiae* when humans are not readily accessible [15]. The authors showed a strong difference between host-seeking preferences (88% antropophilic rate observed in an Odour Baited Entry Trap, OBET) and the real blood meals analysed in blood fed *An. gambiae s.s.* females collected in the same village (half of the blood meals were taken on cattle). Scaling up LLIN coverage may also have strong impact on the distribution and diversity of vector species and then on malaria transmission. In Kenya, authors reported a shift in malaria vector species (*An. arabiensis* replaced *An. gambiae s.s.*) after an increase in LLINs ownership [16]. These issues are now in the spotlight and become a priority in the research agenda as such behavioural modifications may have severe implications for the success of vector control programmes [7].

In the present study, we investigated whether the host seeking behaviour of the major malaria vector *An. funestus* may be modified after the implementation of universal coverage of LLINs. In Benin, *Anopheles gambiae s.s.* populations are strongly resistant to pyrethroid insecticides [10, 17, 18] whereas no pyrethroid resistance was found in *An. funestus* [19]. To avoid any confounding effect link to the presence of pyrethroid resistance alleles, cross-sectional surveys were carried out in two villages (Lokohouè and Tokoli) where *An. funestus* was found predominant and responsible for malaria transmission [19, 20].

Methods

Study Area

This study was carried out in the District Of Ouidah (DOO, Figure 1) in southern Benin (on the Atlantic coast). The local climate is coastal-guinean with four seasons including a long dry season (between November and April). Investigations were conducted in Tokoli (6°26'57.1"

N, 2°09'36.6" E) and Lokohouè (6°24'24.2" N, 2°10'32.1" E) where *An. funestus* is the main malaria vector [19, 20].

Mosquito collection

Indoor and outdoor mosquito collections were done at four sites per villages using the human landing catches (HLC) technique (8 collectors per village per night of collection). Sites were distant from 50 meters minimum and were homogeneously distributed in the village (sites situated near eucalyptus tree, smokes, etc. were discarded) [21]. Collectors were hourly rotated along collection sites and/or position (indoor/outdoor). At each position, all mosquitoes caught were kept in individual tubes and in hourly bags. Independent staff supervised rotations and regularly checked quality of the mosquito collections on a randomly selected sample representing 12% of the total night-collection.

Study design

Three rounds of mosquito collection were done in Tokoli and Lokohouè to study the biting behaviour of malaria vectors. The study design is summarized in the Figure 2.

Round 1 (from October 2007 to May 2008) corresponded to a baseline period of mosquito collection where LLIN (i.e. Permanet® 2.0 containing 55 mg/m2 deltamethrin, Vestergaard Frandsen, Geneva) were provided selectively to pregnant women and children <6 years by the National Malaria Control Programme (NMCP).

Round 2 (from November 2008 to June 2009) corresponded to a period of mosquito collection carried out 1 year after distribution of PermaNet® 2.0 to the entire community (universal coverage) by our team (see [11] for details). Each household was provided with two nets.

Rounds 1 and 2 consisted in five surveys of two consecutive nights (16 human-nights per village per survey) at six week interval. The collection time was between 22:00 and 06:00.

Round 3 (April 2011) corresponded to a period of mosquito collection carried out 3 years following the universal coverage of LLIN. The mosquito collection was done by doing two

surveys of three consecutive nights (24 human-nights per village per survey) at one week interval. The collection period was between 23:00 and 09:00.

Identification of vector species and infection rates

Malaria vectors collected on humans were identified using morphological keys [6, 22]. All mosquitoes belonging to the Funestus Group were kept in individual tubes containing silica gel and preserved at -20°C in the laboratory. Members of the Funestus group were identified to species by PCR using the method describe by Koekemoer *et al.* [23]. Heads and thoraces of *An. funestus* complex were processed for detection of circumsporozoite protein (CSP) of *Plasmodium falciparum* sporozoites using ELISA technique [24].

Entomological indicators

Human Biting Rates (HBR) for *An. funestus* were calculated as numbers of bites per human per night. Sporozoite Rates (SR) were the proportions of *An. funestus* found to be positive for CSP antigens. Entomological Inoculation Rates (EIR, number of infected bites per human per day) was obtained by multiplying the HBR by SR.

WHO bioassays

Susceptibility of *An. funestus* to deltamethrin was checked on mosquitoes collected in Tokoli and Lokohouè by HLC in January 2010. Mosquitoes were kept in cages and brought back to the Centre de Recherche Entomologique de Cotonou (CREC) for rearing. Females were fed on rabbit to obtain eggs (F1 progeny) and larvae were maintained in plastic bowl containing distilled water and dry cat food until adult emergence. Prior bioassays, forty females were randomly selected for identification of sibling species as described above. The other part was tested for pyrethroid susceptibility using the WHO susceptibility tests [25]. Four batches of 25 field-caught, non blood-fed, 2-5 days-old females were exposed to deltamethrin 0.05% treated paper for 1 hour. Two batches of 25 mosquitoes were exposed to untreated paper to serve as a control. Insecticide papers were obtained from the WHO reference centre at the Vector

Control Research Unit, University Sains Malaysia [26]. In the absence of susceptible reference strain of *An. funestus*, the susceptible Kisumu strain of *An. gambiae* (n=100) was exposed to deltamethrin 0.05% treated paper for validation. Percentage of Knocked down (KD) mosquitoes was recorded at 60 minutes after which mosquitoes were held for 24 hours at 27 ± 2°C and 80 ± 10% Relative Humidity. Mortality was recorded 24 hours post-exposure.

Statistical analysis

In order to compare hourly aggressiveness of *An. funestus* before and after implementation of universal LLIN coverage, a Median Catching Time (MCT) was estimated from field data. MCT represents the time for which 50% of the total malaria vectors were caught on humans. MCTs were compared between rounds of collection (pair wise comparisons) using Mann-Whitney *U* tests. Proportions of outdoor biting mosquitoes (exophagy) were compared between rounds of collection in each village using Fisher's exact tests.

SR in *An. funestus* were compared between outdoor and indoor biting vectors, between rounds and between villages using Fisher's exact tests. Odds-ratio and their 95% confidence interval were also calculated.

Ethics statement

The IRD (Institut de Recherche pour le Développement) Ethics Committee and the National Research Ethics Committee of Benin approved the study (CNPERS, reference number IRB00006860). All necessary permits were obtained for the described field studies. No mosquito collection was done without the approval of the head of the village, the owner and occupants of the collection house. Mosquito collectors gave their written informed consent and were treated free of charge for malaria presumed illness throughout the study.

Results

Vector densities and transmission

During the three rounds of HLC collection (i.e. 416 human-nights), 1,866 members of the Funestus Group and 367 specimens belonging to the *An. gambiae* complex were caught. The 1,866 specimens of the Funestus Group processed by PCR for species identification were *Anopheles funestus*. The HBR for all rounds for *An. funestus* was 4.49 bites per person per night. Minimum (2.1 bites/man/night) and maximum HBR (18.73 bites/man/night) were found in Lokohouè at round 1 and 3 respectively.

Twenty-nine *An. funestus* (of 1,866) were found positive for the presence of *P. falciparum* by CSP-ELISA, hence corresponding to a prevalence of *P. falciparum* infection of 1.6%. The EIR for all rounds was 0.06 infected bites of *An. funetus* per person per night. Maximum EIR was found in Lokohouè during round 3 (0.25 infected bites/man/night). All data related to *An. funestus* HBR and EIR at each location and for each round of collection are summarised in Table 1.

We were not able to find any significant difference in SR between rounds of collection (Supplementary Table 1). Moreover, we did not find any significant difference when we compared SR between outdoor and indoor biting *An. funestus* whatever the round or village considered. The same was true when we compared the proportion of infected mosquitoes before and after 06:00 during the round 3.

Biting behaviour

Figure 3 shows the hourly biting aggressiveness of *An. funestus* at each round of collection in Tokoli and Lokohouè. During round 1 (i.e. selective coverage of LLIN), the peak of aggressiveness of *An. funestus* was between midnight and 01:00 in Tokoli (Figure 3A). During round 2 (i.e. 1 year after implementation of universal coverage of LLIN), we observed two peaks of activity in the same village: the first peak was similar to round 1 (between 00:00

and 01:00) but the second peak was reported later during the night (between 03:00 and 04:00; Figure 3C). The analysis of Median Catching Time (MCT) showed a significant difference between the round 1 and 2 (figure 4A; Mann-Whitney *U* test p-value = 0.0028). During the round 3 (i.e. 3 years after universal coverage of LLIN), only one peak of activity was observed between 4h and 6h (Figure 3E) and the MCT was 05:00, later than that recorded in the previous rounds of collection (Figure 4A; Mann-Whitney *U* test p-value = 0.0039). Between 2008 and 2011, the MCT in *An. funestus* population switched from 02:00 to 05:00 in the morning (Mann-Whitney *U* test p-value <0.0001).

In Lokohouè, we were not able to identify a peak of activity during the round 1 (Figure 3B). However, we clearly observed a peak of aggressiveness just before dawn (from 05:00 to 06:00) during rounds 2 and 3 (Figure 3D and 3F). The MCT was 03:00 before full coverage of LLIN (Figure 4B) and it shifted to 04:00 and 05:00 during round 2 and 3 respectively (Mann-Whitney *U* test p-value <0.0001).

During the round 3, 26.4 % of the overall *An. funestus* were caught after 06.00 h in Lokohouè, (Table 2) whereas the proportion of late biting mosquito was 6.6 % in Tokoli (OR=5.084 95CI 2.63-9.82; p<0.0001). The morning civil dawn (i.e. the beginning of twilight) was 06:17 during the round 3 of collection.

Regarding exophagy rates, the proportion of outdoor biting mosquitoes was similar in Tokoli and Lokohouè during the round 1 (45.6 and 44.6 % respectively, Table 3).

In Tokoli, exophagy increased significantly to 68.1 % (OR=2.55 95CI 1.72-3.78, p<0.0001) and 60.9 % (OR=1.86 95CI 1.21-2.85, p=0.0052) during the rounds 2 and 3 respectively whereas it remained unchanged in Lokohouè (44.2 % at round 2, p=1 and 46.7 % at round 3, p=0.6737).

Resistance to insecticides

Bioassays showed that females of *An. funestus* were fully susceptible to deltamethrin (100% mortality). Moreover, mosquitoes were 100% knocked-down (KD) after 60 minutes exposure suggesting the absence of any knockdown resistance (kdr) alleles. Hundred exposed mosquitoes of the susceptible strain Kisumu of *An. gambiae* showed 100% mortality and 100% KD. No mortality was observed in the control tubes (i.e. with untreated paper). Among the 40 specimens checked by PCR for species identification, all belonged to *Anopheles funestus*.

Discussion

This study reported significant changes in the host seeking behaviour of the *Anopheles funestus* population after scaling up universal coverage of LLIN in southern Benin. Results showed that 3 years after implementation of LLIN at community level, *Anopheles funestus* bit later in the night (almost at dawn) and more frequently outdoor compared to the baseline survey. Induced exophagy and late-biting behaviour were already observed in African malaria vectors after implementation of indoor residual spraying [12, 27]. In Benin, the results of a randomized control trial conducted in 28 villages showed that the prevalence of outdoor biting malaria vectors was higher in villages covered by the combination of LLIN and carbamate IRS compared with LLIN alone [11]. Regarding *An. funestus,* recent findings showed a shift from indoor to outdoor biting in Tanzania [14] in relation with increasing coverage of pyrethroid impregnated nets. However, authors showed a shift of biting time of *An. funestus* to the early evening and not late in the morning as we observed in the present study. To our knowledge, very few studies have reported a peak of aggressiveness of *An. funestus* during the last hour of collection, prior to dawn [28-30]. One of them [28] was in northern Ghana in

a context of nationwide distribution of LLIN but the relationship between mosquito behaviour and vector control method could not be clearly established.

Changes in mosquito's feeding behaviour can be associated with seasonality [31]. Most cited environmental factors influencing the biting habits of mosquitoes are wind, rain and temperature [32]. Usually, wind and rain occur simultaneously in tropical storms and can drastically reduce the number of mosquitoes caught on humans. However, we never conducted any mosquito collection when the weather was bad. Moreover, we observed that nocturnal temperatures were not different between rounds of collection nor correlated with changes in biting behaviour (see in the Supplementary Data file, the table 2 and table 3). This suggests that local climatic conditions were unlikely to be responsible for the switch in *An. funestus* biting behaviour during the study.

Here, we provide the first evidence for a substantial diurnal host-biting behaviour of a major malaria vector in Africa. Indeed, during the round 3 in Lokohouè, a large proportion of the aggressive fraction of *An. funestus* (26%) was collected after 06:00. It is important to note that in both villages during round 1, the proportion of night's biting of *An. funestus* between 05:00 and 06:00 was higher than 10 % suggesting that a diurnal biting activity was already present before the implementation of LLIN. The dogma that malaria vectors are strictly nocturnal may be not entirely true, especially if they have been exposed to intense selection pressure due to the scaling up of residual insecticide for malaria vector control. Moreover, in many studies where the peaks of aggressiveness of *An. funestus* occurred during the last hours of collection (before dawn) [28, 29, 33], the estimation of malaria transmission might have been under-estimated. Biting preferences of malaria vectors will have to be more frequently investigated after dawn in different ecological settings. The late and outdoor biting behaviour of malaria vector is worrying because in rural Africa, villagers usually wake up before dawn to work in crops and as such they are not protected by mosquito nets. This might explain why

malaria prevalence or incidence remained high despite the high LLIN coverage in areas where *An. funestus* is the dominant malaria vector [3, 28, 34]. Moreover, *An. funestus* may play an important role in malaria transmission during the dry-hot season [6, 35-37], when LLINs are less likely to be used due to high nocturnal temperatures and low mosquito biting nuisances [20].

Interestingly, strong increase of outdoor biting mosquitoes was observed in Tokoli where the proportion of vector biting after 06:00 was the lowest one. This contrasts with the situation in Lokohouè where lower exophagy rates but higher late morning biting rates were observed. These findings raise crucial questions about the evolutionary processes involved in mosquito behaviour in relation with insecticide treatments. Beyond the dogma of the strict nocturnal biting activity of the African malaria vectors, there is a consensus for a trade-off between the energy gain acquired through the blood meal and the risk caused by the defensive behaviour of the host [31, 38]. Recent but massive selection pressure induced by vector control tools may have altered the human-vector interactions. It is therefore interesting to note that one behaviour among late biting and outdoor biting predominated in each village suggested that vector control interventions may select for different adaptative responses and probably genetic diversity among vector populations. Clearly, there is an urgent need to better understand the evolution processes involved in host-seeking in malaria vectors in relation to vector control tools [7, 8].

Insecticide resistance is frequently questioned in vector control failure relying on residual insecticides [10]. The resistance mechanisms that allow mosquitoes to survive to insecticides might influence behavioural traits. Here, the *An. funestus* population was fully susceptible to deltamethrin, the insecticide used in Permanet® 2.0. Thus, modifications of biting behaviour observed after full coverage of LLINs cannot be attributed to pleiotropic effects or to the

presence of any pyrethroid resistance mechanism. Adaptation of *An. funestus* to LLIN may result from a phenotypic plasticity or to selected behavioural traits. In Senegal and in Burkina Faso [39, 40], chromosomal forms of *An. funestus* were found to be associated with different resting, biting or host preference behaviour. We assume that in southern Benin, a genetically distinct form of *An. funestus* might be selected by vector control interventions. Further investigations in cytogenetic, population genetics and mosquito behaviour are however required to confirm this trend.

In conclusion, we found evidence for a modulation of *An. funestus* biting behaviour following implementation of full coverage of LLINs at community level. Vectors biting outdoor and/or at dawn when people are not longer protected by a residual insecticide (LLIN or IRS) is worrying for malaria prevention in Africa. These findings highlighted the need for new vector control strategies to better interrupt outdoor and diurnal malaria transmission.

Permission:

We provide written permission for all personal communications

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Figures:

Figure 1 - Map of the study area

Normalised Difference Vegetation Index (NDVI) was calculated from SPOT (Satellite Pour l'Observation de la Terre) data, ©CNES (2010), Distribution Spot Image S.A.

Freshwater (included Toho Lake) is shown as dark grey. Healthy vegetation is shown as light grey and white.

Figure 2 - Chronogram presenting Long Lasting Insecticidal impregnated Nets (LLIN) distributions and Human Landing Catch (HLC) during the study.

Figure 3 - Hourly biting activity of *An. funestus* in Tokoli (A,C,E) and Lokohouè (B,D,F) before and after implementation of universal coverage of LLIN.

Vertical grey lines indicate morning civil dawn.

Figure 4 – Median Catching Time of *An. funestus* before and after implementation of universal coverage of LLIN in Tokoli (A) and Lokohouè (B).

Boxes indicate 1^{st}-3^{rd} quartile and median hours of biting activity. Whiskers indicate 2.5-97.5 percentiles. Boxes carrying the same letter were not significantly different ($p<0.05$) when comparing median catching time using Mann-Whitney U tests. In order to compare all rounds to each other, only mosquitoes caught between 23h and 6h were taken into account in the stastical analysis.

Tables

Table 1 - Aggressiveness and Entomological Inoculation Rates of *Anopheles funestus* before and after implementation of universal coverage of LLIN.

HBR : Human Biting Rate, number of bites/man/night. SR: Sporozoite Rate, proportion of vectors positive to CSP antigenes. EIR: Entomological Inoculation Rate, number of infected bites/man/night.

	Time of catch	Month of collection	No. of human-night	No. bites of *An. funestus*	HBR	SR (%)	EIR
Tokoli							
Round 1 (Baseline)	22 to 6	Oct. to May	80	204	2.55	1.96	0.05
Round 2	22 to 6	Nov. to Jun.	80	226	2.83	0.88	0.03
Round 3	23 to 9	Apr.	48	152	3.17	3.29	0.10
Lokohouè							
Round 1 (Baseline)	22 to 6	Oct. to May	80	168	2.10	2.38	0.05
Round 2	22 to 6	Nov. to Jun	80	217	2.71	0.92	0.03
Round 3	23 to 9	Apr.	48	899	18.73	1.34	0.25

Table 2 – Rates of *Anopheles funestus* biting after 6h, three years after implementation of universal coverage of LLIN.

CI: Confidence interval; Odds-ratio, 95%CI and p-value according to a Fisher exact test.

	No. of bites before 6h	No. of bites after 6h	After 6h rate (%)	Odds-Ratio	95% CI	P-Value
Tokoli	142	10	6.6	1		
Lokohouè	662	237	26.4	**5.084**	2.632 to 9.820	**<0.0001**

Table 3 - Proportion of *Anopheles funestus* biting outdoor before and after implementation of universal coverage of LLIN.

CI: Confidence interval; Odds-ratio, 95%CI and p-value according to Fisher exact tests.

	No. of outdoor bites	No. of indoor bites	Rate of exophagy (%)	Odds ratio	95% CI	p-value
Tokoli						
Round 1 (Baseline)	93	111	45.6	1		
Round 2	154	72	68.1	**2.553**	1.724 to 3.781	**<0.0001**
Round 3	92	59	60.9	**1.861**	1.214 to 2.854	**0.0052**
Lokohouè						
Round 1 (Baseline)	75	93	44.6	1		
Round 2	96	121	44.2	0.9838	0.6559 to 1.476	1
Round 3	419	479	46.7	1.071	0.7788 to 1.511	0.6737

Figure1

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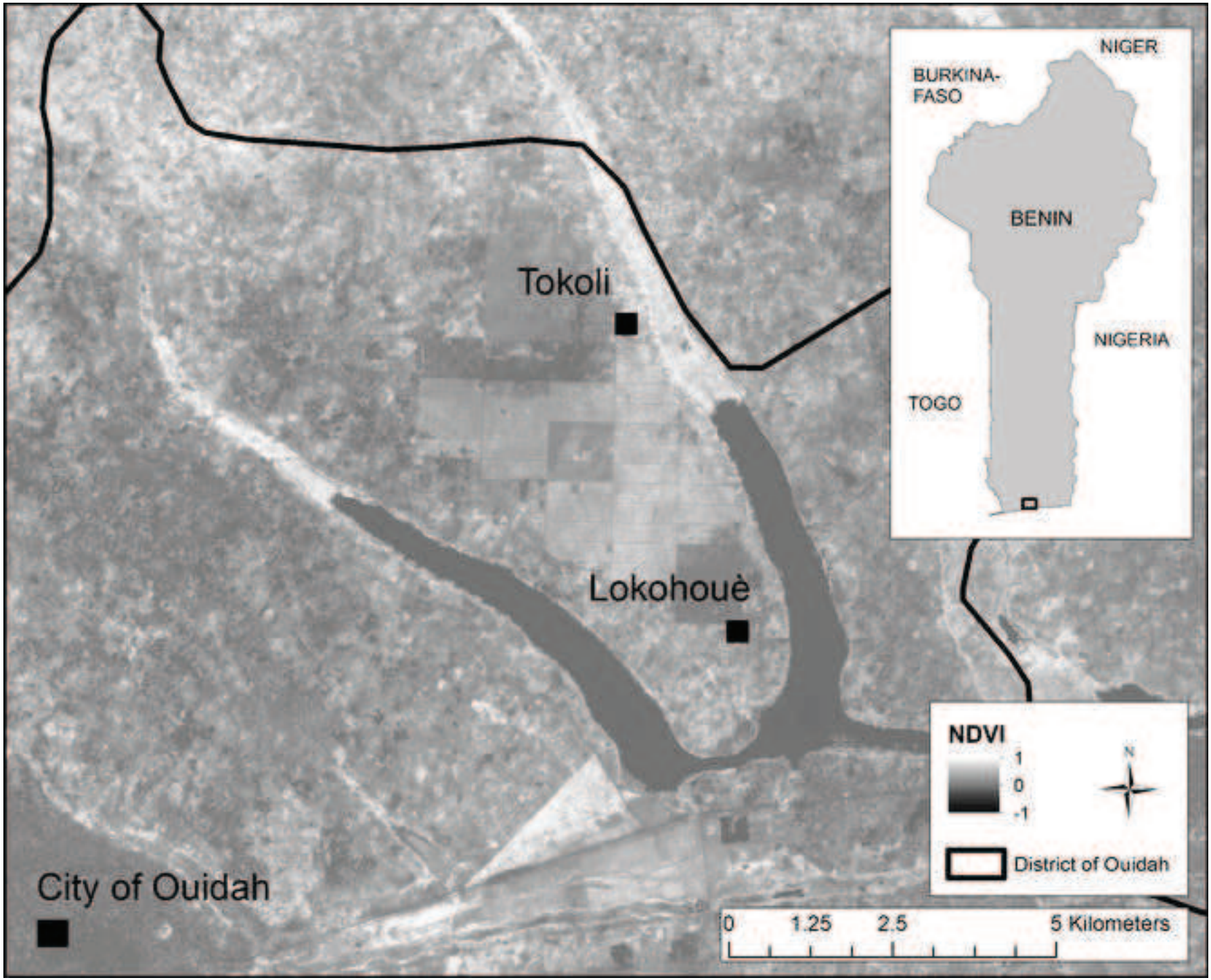

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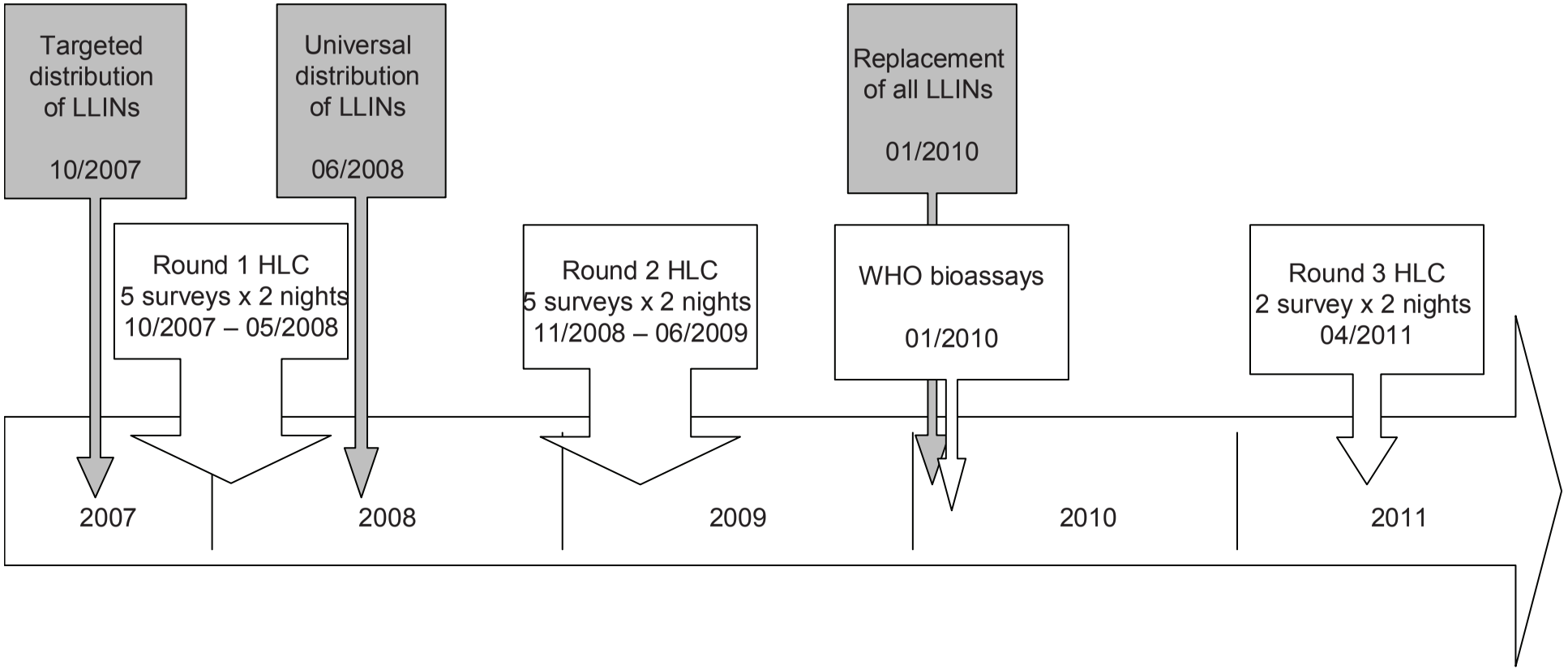

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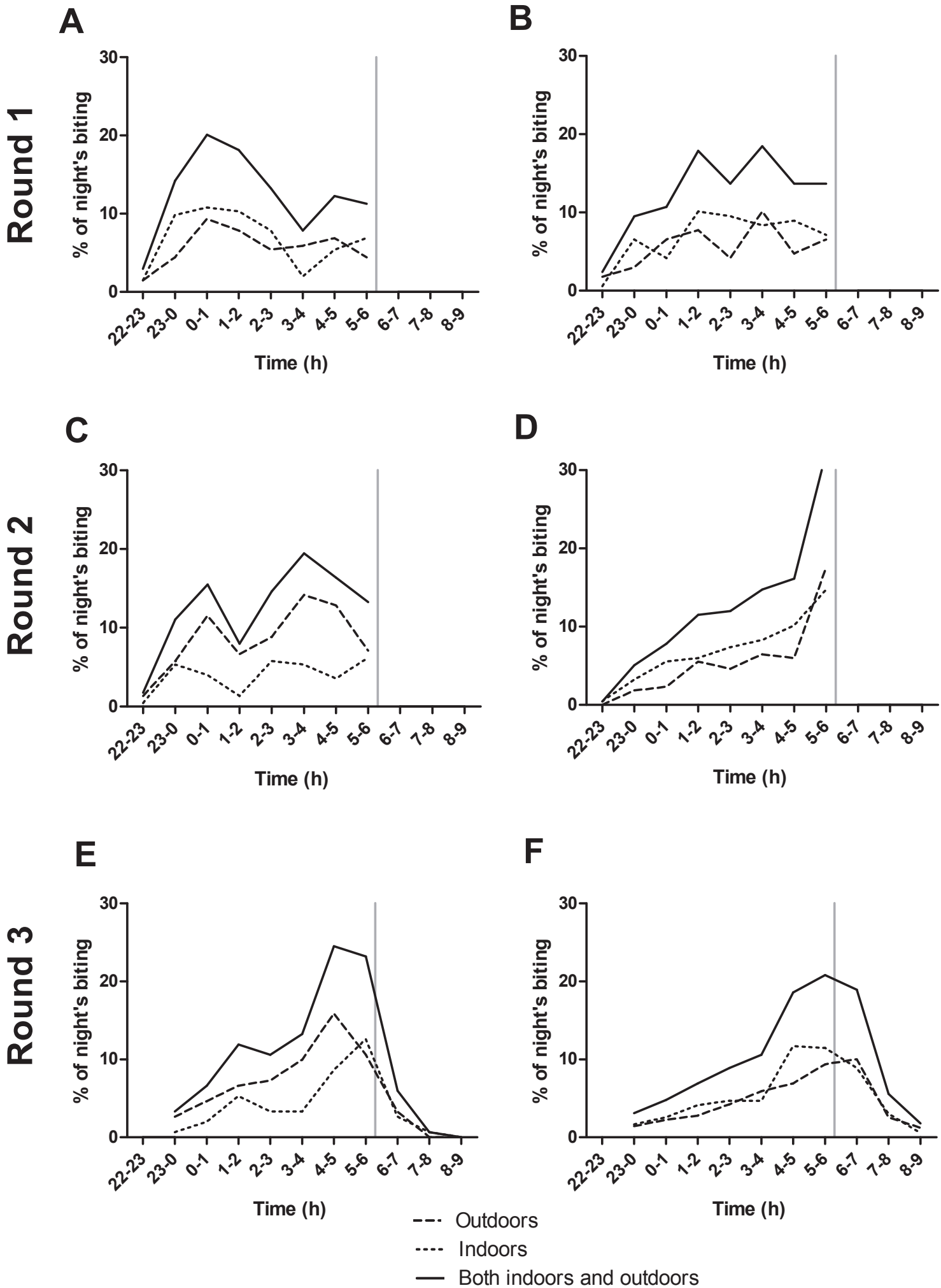

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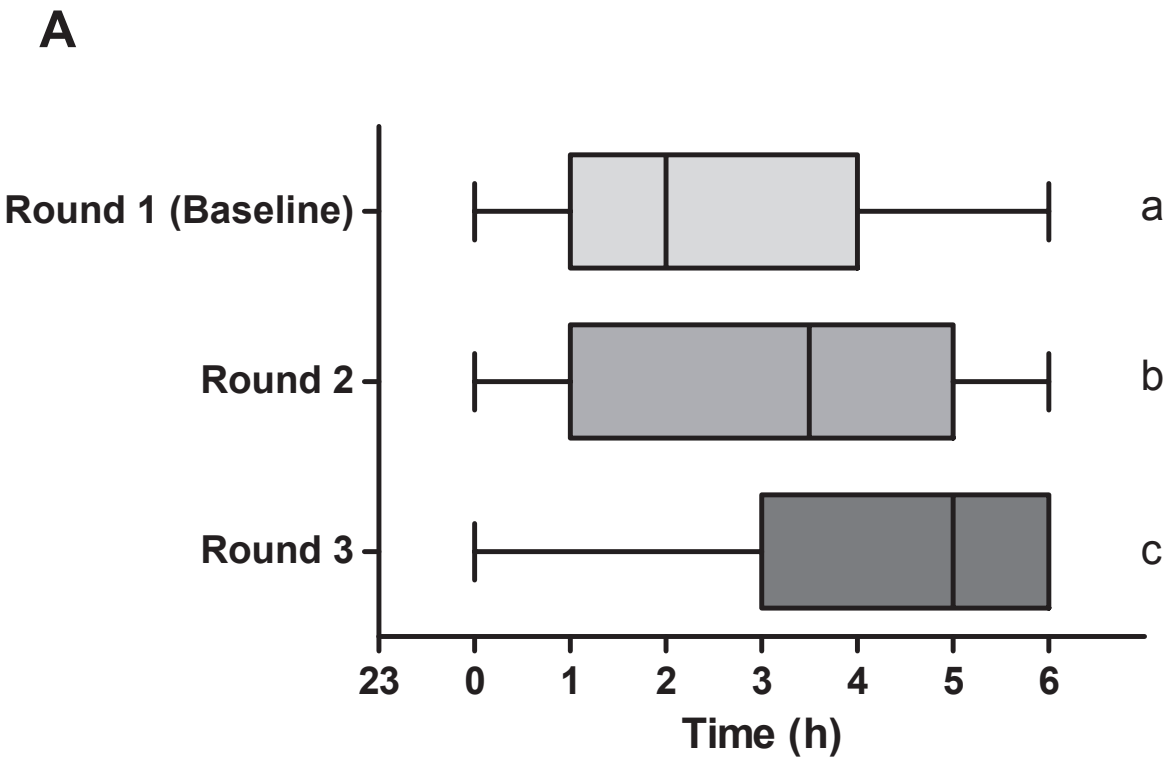

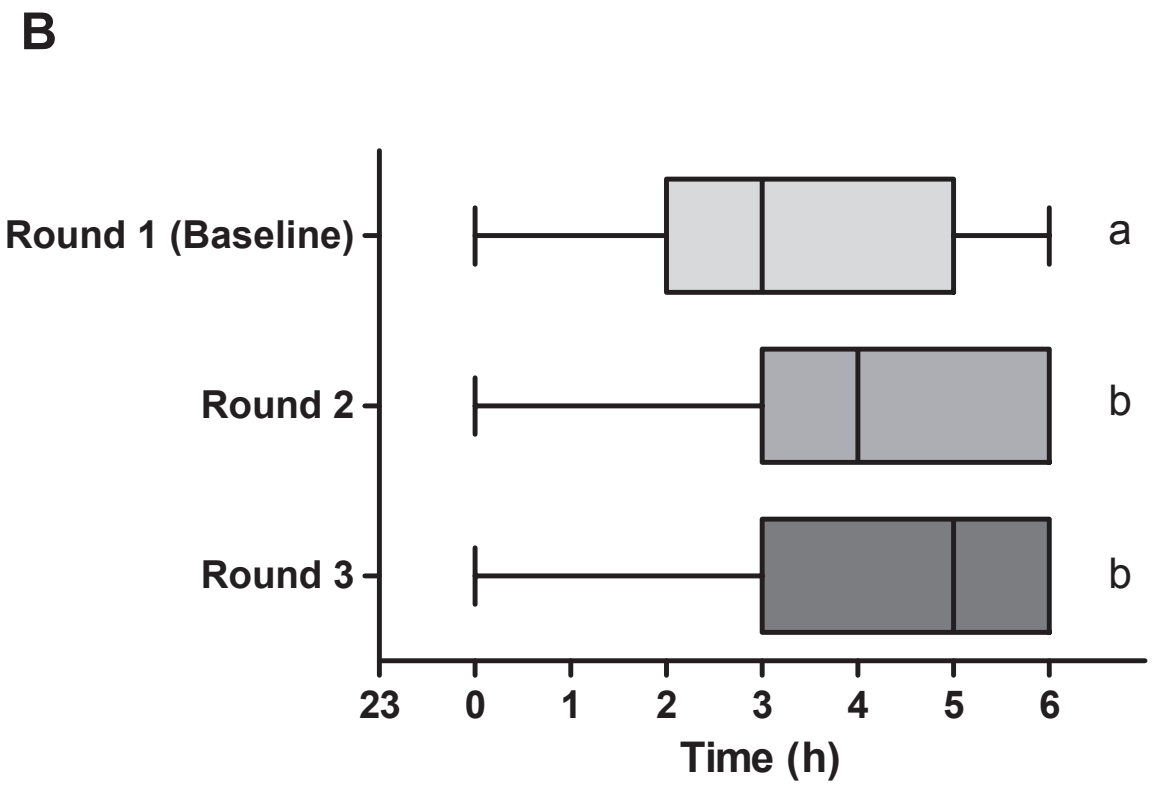

Supplementary Methods:

We used the Land-Surface Temperature (LST) at a spatial resolution of one kilometer measured by the Moderate Resolution Imaging Spectroradiometer (MODIS) sensors on the Terra satellite (https://lpdaac.usgs.gov/). The average 8-day nocturnal temperature during each survey at the coordinates of each village (georeferenced using the Global Positioning System) was extracted using ArcGis ArcInfo 9.3 software (ESRI, Redlands, CA). The data were then converted into Celsius.

In order to assess the combined affect of the nocturnal temperatures, the village and the vector control intervention implemented on the biting behavior of *An. funestus*, we performed multivariate analyses of the exophagy rate and the biting cycle.

The Proportion of Outdoor Biting (POB) *An. funestus* during each survey was assessed using a Generalized Linear Model (GLM) with a binomial distribution. The proportion of *An. funestus* caught during the second part of the night (i.e. after 03:00) and during each survey was assessed using a GLM with a binomial distribution. In order to compare all rounds to each other, only mosquitoes caught between 23:00 and 6:00 were taken into account.

Supplementary Results:

Both in Lokohoué and Tokoli, we did not find significant differences of temperature between the round 1 and 2. Mean nocturnal temperatures were 20.98°C (95CI 17.49-24.47) during round 1 and 20.96°C (95CI 17.4-24.51; T-test p-value = 0.989) during round 2 in Tokoli. Mean nocturnal temperatures were 22.13°C (95CI 18.55-25.71) during round 1 and 22.52°C (95CI 19.97-25.06; T-test p-value = 0.813) during round 2 in Lokohouè. During round 3, mean nocturnal temperatures were 21.56°C in Tokoli and 21.52°C in Lokohouè.

The GLM analysis of the POB showed that the nocturnal temperatures did not explain a significant part of the deviance (OR=0.99, CI95 0.94-1.05, p=0.8; Supplementary Table 2). POB was significantly higher in Tokoli during rounds 2 (OR=2.55, CI95 1.72-3.78, $p<10^{-4}$) and 3 (OR=1.88, CI95 1.22-2.90, p=0.004). In contrast, there was not significant effect of the round in Lokohouè (since rounds effects and interaction terms between rounds and Lokohouè canceled each other out). This indicates that *An. funestus* bites more outdoor in Tokoli after implementation of the universal coverage with LLINs.

The nocturnal temperatures did not explain a significant part of the deviance of the proportion of biting *An. funestus* caught after 03:00 (OR=1.01, CI95 0.97-1.06, p=0.61; Supplementary Table 3). The proportion of *An. funestus* biting during the second part of the night was also higher in Lokohouè (OR=1.24, CI95 1.01 1.52, p=0.04) than in Tokoli. This proportion was higher during round 2 (OR=2.03, CI95 1.53-2.69, $p<10^{-5}$) and 3 (OR=3.43, 2.67-4.39, $p<10^{-15}$) than during round 1, indicating that the distribution of biting *An. funestus* switched to the last hours of the night. We observed the same results even when other thresholds (i.e. 00:00, 01:00, 02:00, 04:00 or 05:00) were applied (data not shown).

Supplementary Tables:

Supplementary Table 1 – Sporozoite Rates of *Anopheles funestus* according to the village, rounds, time of collection and indoor or outdoor seat.

CI: Confidence interval; Odds-ratio, 95%CI and p-value according to a Fisher exact test. CSP -/+: number of *An. funestus* found negative/positive for the CSP antigen. /: indicates that both modalities (i.e. both villages, both seats or both periods of collection) were taken into account.

Village	Round	Seat	Time of collection	CSP -	CSP +	SR	Odds-Ratio	95 CI	P-value
Tokoli	Round 1	Outdoor	/	91	2	2.15%	1		
		Indoor	/	109	2	1.80%	0,8349	0.1153 to 6.047	1
	Round 2	Outdoor	/	153	1	0.65%	1		
		Indoor	/	71	1	1.39%	2,155	0.1328 to 34.97	0.5366
	Round 3	Outdoor	/	91	1	1.09%	1		
		Indoor	/	55	4	6.78%	6,618	0.7208 to 60.77	0.0766
Lokohouè	Round 1	Outdoor	/	74	1	1.33%	1		
		Indoor	/	90	3	3.23%	2,467	0.2512 to 24.23	0.6292
	Round 2	Outdoor	/	95	1	1.04%	1		
		Indoor	/	120	1	0.83%	0,7917	0.04884 to 12.83	1
	Round 3	Outdoor	/	413	4	0.96%	1		
		Indoor	/	471	8	1.67%	1,754	0.5241 to 5.868	0.3984
/	Round 3	/	Before 06h00	893	16	1.76%	1		
		/	After 06h00	264	4	1.49%	0,8456	0.2802 to 2.552	1
Tokoli	Round 1	/	/	200	4	1.96%	1		
	Round 2	/	/	224	2	0.88%	0.4464	0.08087 to 2.465	0.4293
	Round 3	/	/	146	5	3.31%	1.712	0.4518 to 6.489	0.5036
Tokoli	Round 2	/	/	224	2	0.88%	1		
	Round 3	/	/	146	5	3.31%	3.836	0.7341 to 20.04	0.1215
		/	/						
Lokohouè	Round 1	/	/	164	4	2.38%	1		
	Round 2	/	/	215	2	0.92%	0.6491	0.3643 to 1.156	0.4104
	Round 3	/	/	884	12	1.34%	0.5566	0.1773 to 1.747	0.2998

Lokohouè	Round 2	/	/	215	2	0.92%	1		
	Round 3	/	/	884	12	1.34%	1.459	0.3241 to 6.571	1.0000

Supplementary Table 2: Multivariate analysis of the Proportion of *An. funestus* Biting Outdoor.

SE: Standard error of the estimates. 95 CI: 95% Confidence Interval of the Odds-Ratio.

Effects	Estimates	SE	Odds-Ratio	95 CI		p-value	
Nocturnal temperatures	-0.01	0.03	0.99	0.94	1.05	0.7976	
Round 1			1				
Round 2	0.94	0.20	2.55	1.72	3.78	0.0000	***
Round 3	0.63	0.22	1.88	1.22	2.90	0.0044	**
Tokoli			1				
Lokohouè	-0.03	0.21	0.97	0.64	1.48	0.8990	
Round 2 : Lokohouè	-0.95	0.29	0.39	0.22	0.68	0.0011	**
Round 3 : Lokohouè	-0.55	0.28	0.58	0.33	1.00	0.0484	*

Supplementary table 3: Multivariate analyses of the proportion of *An. funestus* biting after 03:00h.

SE: Standard error of the estimates. 95 CI: 95% Confidence Interval of the Odds-Ratio.

Effects	Estimates	SE	Odds-Ratio	95 CI		p-value	
Nocturnal temperatures	0.01	0.02	1.01	0.97	1.06	0.61	
Round 1			1				
Round 2	0.71	0.14	2.03	1.53	2.69	1.09E-006	***
Round 3	1.23	0.13	3.43	2.67	4.39	< 2e-16	***
Tokoli			1				
Lokohouè	0.21	0.1	1.24	1.01	1.52	0.04	*