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

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**Background.** Behavioral 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 (LLINs) may induce a switch in biting behavior in *Anopheles funestus*, a major malaria vector in Africa.

**Methods.** Human-landing collections were conducted indoor and outdoor in 2 villages (Lokohouè and Tokoli) in Benin before and 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 *A. funestus* to deltamethrin was monitored using bioassays.

**Results.** MCT of *A. funestus* switched from 2 AM in Lokohouè and 3 AM in Tokoli to 5 AM after 3 years (Mann–Whitney U test, *P* < .0001). In Tokoli, POB increased from 45% to 68.1% (odds ratio = 2.55; 95% confidence interval = 1.72–3.78; *P* < .0001) 1 year after the universal coverage, whereas POB was unchanged in Lokohouè. In Lokohouè, however, the proportion of *A. funestus* that bites after 6 AM was 26%. Bioassays showed no resistance to deltamethrin.

**Conclusions.** This study provides evidence for a switch in malaria vectors’ biting behavior after 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.

During the past 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 behavioral ecology of the main malaria vectors in Africa, *Anopheles gambiae* and *Anopheles funestus* [6]. Both interventions target vectors when they feed and/or rest indoors [7]. However, as described by Fergusson et al [8], there are many ecological reasons for all vectors to not be targeted by an insecticide (eg, insecticide resistance, behavioral avoidance, and vector biodiversity). 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 behavioral changes of mosquitoes after the implementation of vector control interventions [12]. Indeed, recent evidence suggests that malaria vectors may avoid the contact with the insecticide by either feeding predominantly outdoor or in the early evening.
This behavioral 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 behavior in *A. gambiae* when humans are not readily accessible [15]. The authors showed a strong difference between host-seeking preferences (88% anthropophilic rate observed in an odor-baited entry trap) and the real blood meals analyzed in blood-fed *A. gambiae* s.s. females collected in the same village (half 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 (*A. arabiensis* replaced *A. gambiae* s.s.) after an increase in LLIN ownership [16]. These issues are now in the spotlight and have become a priority in the research agenda, because such behavioral modifications may have severe implications for the success of vector control programs [7].

In the present study, we investigated whether the host-seeking behavior of the major malaria vector *A. funestus* may be modified after the implementation of universal coverage of LLINs. In Benin, *A. gambiae* s.s. populations are strongly resistant to pyrethroid insecticides [10, 17, 18], whereas no pyrethroid resistance was found in *A. funestus* [19]. To avoid any confounding effect of the presence of pyrethroid resistance alleles, cross-sectional surveys were performed in 2 villages (Lokohouè and Tokoli) where *A. funestus* was found to be predominant and responsible for malaria transmission [19, 20].

**METHODS**

**Study Area**

This study was performed in the District Of Ouidah (Figure 1) in southern Benin (on the Atlantic coast). The local climate is coastal-guinean, with 4 seasons including a long dry season (from November through 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 *A. funestus* is the main malaria vector [19, 20].

**Mosquito Collection**

Indoor and outdoor mosquito collections were done at 4 sites per village with use of 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 trees and smokes, for example, were discarded) [21]. Collectors were hourly rotated along collection sites and/or position (indoor or 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.

**Figure 1.** Map of the study area. Normalised difference vegetation index (NDVI) calculated from 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.
Study Design

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

Round 1 (from October 2007 through May 2008) corresponded to a baseline period of mosquito collection during which LLINs (ie, Permanet 2.0, containing 55 mg/m² deltamethrin; Vestergaard Frandsen) were provided selectively to pregnant women and children aged <6 years by the National Malaria Control Programme.

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

Rounds 1 and 2 consisted of 5 surveys of 2 consecutive nights (16 human-nights per village per survey) at 6-week intervals. The collection time was 10 PM to 6 AM.

Round 3 (April 2011) corresponded to a period of mosquito collection performed 3 years after universal coverage of LLINs. Mosquito collection was done by conducting 2 surveys on 3 consecutive nights (24 human-nights per village per survey) at 1-week intervals. The collection period was 11 PM to 9 AM.

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 were preserved at -20°C in the laboratory. Members of the funestus group were identified to species by polymerase chain reaction (PCR) with use of the method described by Koekemoer et al [23]. Heads and thoraces of A. funestus complex were processed for detection of circumsporozoite protein (CSP) of Plasmodium falciparum sporozoites with use of enzyme-linked immunosorbent assay (ELISA) technique [24].

Entomological Indicators

Human biting rates (HBRs) for A. funestus were calculated as numbers of bites per human per night. Sporozoite rates (SRs) were the proportions of A. funestus found to be positive for CSP antigens. Entomological inoculation rates (EIRs; number of infected bites per human per day) were obtained by multiplying the HBR by SR.

World Health Organization (WHO) Bioassays

Susceptibility of A. 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 for rearing. Females were fed on rabbit to obtain eggs (F1 progeny), and larvae were maintained in a plastic bowl containing distilled water and dry cat food until adult emergence. Before bioassays were performed, 40 females were randomly selected for identification of sibling species, as described above. The other part was tested for pyrethroid susceptibility with use of the WHO susceptibility tests [25]. Four batches of 25 field-caught, non–blood-fed, 2–5-day-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 center at the Vector Control Research Unit, University Sains Malaysia [26]. In the absence of susceptible reference strain of A. funestus, the susceptible Kisumu strain of A. 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.
relative humidity. Mortality was recorded 24 hours after exposure.

**Statistical Analysis**
To compare hourly aggressiveness of *A. 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) with use of Mann–Whitney U tests. Proportions of outdoor biting mosquitoes (exophagy) were compared between rounds of collection in each village with use of Fisher’s exact tests.

SRs in *A. funestus* were compared between outdoor and indoor biting vectors, between rounds and between villages, with use of Fisher’s exact tests. Odds ratios (ORs) and their 95% confidence intervals (CIs) were also calculated.

**Ethics Statement**
The Institut de Recherche pour le Développement Ethics Committee and the National Research Ethics Committee of Benin approved the study (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 and 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 3 rounds of HLC collection (ie, 416 human-nights), 1866 members of the funestus group and 367 specimens belonging to the *A. gambiae* complex were caught. The 1866 specimens of the funestus group processed by PCR for species identification were *A. funestus*. The HBR for all rounds for *A. funestus* was 4.49 bites per person per night. Minimum (2.1 bites/person/night) and maximum HBRs (18.73 bites/person/night) were found in Lokohouè during rounds 1 and 3, respectively.

Twenty-nine of 1866 *A. funestus* were found to be positive for the presence of *P. falciparum* by CSP-ELISA, corresponding to a prevalence of *P. falciparum* infection of 1.6%. The EIR for all rounds was 0.06 infected bites of *A. funetust* per person per night. Maximum EIR was found in Lokohouè during round 3 (0.25 infected bites/person/night). All data related to *A. funestus* HBR and EIR at each location and for each round of collection are summarized in Table 1.

We were not able to find any statistically significant difference in SR between rounds of collection (Supplementary Table 1). Moreover, we did not find any statistically significant difference when we compared SR between outdoor and indoor biting *A. funestus*, regardless of the round or village considered. The same was true when we compared the proportion of infected mosquitoes before and after 6 AM during round 3.

**Biting Behavior**
Figure 3 shows the hourly biting aggressiveness of *A. funestus* during each round of collection in Tokoli and Lokohouè. During round 1 (ie, selective coverage of LLIN), the peak of aggressiveness of *A. funestus* was from midnight to 01 AM in Tokoli (Figure 3A). During round 2 (ie, 1 year after implementation of universal coverage of LLIN), we observed 2 peaks of activity in the same village: the first peak was similar to round 1 (from midnight to 1 AM), but the second peak was reported later during the night (from 03 AM to 04 AM) (Figure 3C). The analysis of MCT showed a statistically significant difference between rounds 1 and 2 (Mann–Whitney U test, *P* = .0028) (Figure 4A). During round 3 (ie, 3 years after universal coverage of LLIN), only 1 peak of activity was observed from 4 AM to 6 AM (Figure 3E), and the MCT was 5 AM,

<table>
<thead>
<tr>
<th>Location, Period</th>
<th>Time of catch</th>
<th>Month of collection</th>
<th>No. of human-nights</th>
<th>No. bites of <em>A. funestus</em></th>
<th>HBR</th>
<th>SR (%)</th>
<th>EIR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tokoli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Round 1 (Baseline)</td>
<td>10 PM to 6 AM</td>
<td>October to May</td>
<td>80</td>
<td>204</td>
<td>2.55</td>
<td>1.96</td>
<td>0.05</td>
</tr>
<tr>
<td>Round 2</td>
<td>10 PM to 6 AM</td>
<td>November to June</td>
<td>80</td>
<td>226</td>
<td>2.83</td>
<td>0.88</td>
<td>0.03</td>
</tr>
<tr>
<td>Round 3</td>
<td>11 PM to 9 AM</td>
<td>April</td>
<td>48</td>
<td>152</td>
<td>3.17</td>
<td>3.29</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>Lokohouè</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Round 1 (Baseline)</td>
<td>10 PM to 6 AM</td>
<td>October to May</td>
<td>80</td>
<td>168</td>
<td>2.10</td>
<td>2.38</td>
<td>0.05</td>
</tr>
<tr>
<td>Round 2</td>
<td>10 PM to 6 AM</td>
<td>November to June</td>
<td>80</td>
<td>217</td>
<td>2.71</td>
<td>0.92</td>
<td>0.03</td>
</tr>
<tr>
<td>Round 3</td>
<td>11 PM to 9 AM</td>
<td>April</td>
<td>48</td>
<td>899</td>
<td>18.73</td>
<td>1.34</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Abbreviations: EIR, entomological inoculation rate (number of infected bites/person/night); HBR, human biting rate (number of bites/person/night); SR, sporozoite rate (proportion of vectors positive to CSP antigens).
later than that recorded during the previous rounds of collection (Mann–Whitney U test, $P = .0039$) (Figure 4A). During 2008–2011, the MCT in *A. funestus* population switched from 2 AM to 5 AM (Mann–Whitney U test, $P < .0001$).

In Lokohouè, we were not able to identify a peak of activity during round 1 (Figure 3B). However, we clearly observed a peak of aggressiveness just before dawn (5–6 AM) during rounds 2 and 3 (Figure 3D and F). The MCT was 3 AM before full coverage of LLIN (Figure 4B), and it shifted to 4 AM and 5 AM during rounds 2 and 3, respectively (Mann–Whitney U test, $P < .0001$).

During round 3, 26.4% of the overall *A. funestus* were caught after 6 AM in Lokohouè, (Table 2), whereas the proportion of late-biting mosquitoes was 6.6% in Tokoli (OR = 5.084; 95% CI, 2.63–9.82; $P < .0001$). The morning civil dawn (ie, the beginning of twilight) was 6:17 AM during round 3 of collection.

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

In Tokoli, exophagy increased significantly to 68.1% (OR = 2.55; 95% CI, 1.72–3.78; $P < .0001$) and 60.9% (OR = 1.86; 95% CI, 1.21–2.85; $P = .0052$) during rounds 2 and 3, respectively, whereas it remained unchanged in Lokohouè (44.2% at round 2 [$P > .99$] and 46.7% at round 3 [$P = .6737$]).
Resistance to Insecticides

Bioassays showed that females of *A. funestus* were fully susceptible to deltamethrin (100% mortality). Moreover, mosquitoes were 100% KD after 60 minutes of exposure, suggesting the absence of any KD resistance alleles. One hundred exposed mosquitoes of the susceptible strain Kisumu of *A. gambiae* showed 100% mortality and 100% KD. No mortality was observed in the control tubes (ie, with untreated paper). Of the 40 specimens checked by PCR for species identification, all belonged to *A. funestus*.

DISCUSSION

This study reported significant changes in the host-seeking behavior of the *A. funestus* population after scaling up universal coverage of LLINs in southern Benin. Results showed that 3 years after implementation of LLIN at community level, *A. funestus* bit later during the night (almost at dawn) and more frequently outdoors, compared with the baseline survey. Induced exophagy and late-biting behavior 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 LLINs and carbamate IRS, compared with LLINs alone [11]. With regard to *A. funestus*, recent findings showed a shift from indoor to outdoor biting in Tanzania [14] in relation to increasing coverage of pyrethroid-impregnated nets. However, the authors showed a shift of biting time of *A. 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 *A. funestus* during the last hour of collection, before dawn [28–30]. One such study [28] was in northern Ghana in the context of nationwide distribution of LLINs, but the relationship between mosquito behavior and vector control method could not be clearly established.

Changes in mosquitoes’ feeding behavior 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

Table 2. Rates of *Anopheles funestus* Biting after 6 AM, Three Years After Implementation of Universal Coverage of Long-Lasting Insecticidal Impregnated Nets (LLINs)

<table>
<thead>
<tr>
<th>Location</th>
<th>No. of bites before 6 AM</th>
<th>No. of bites after 6 AM</th>
<th>After 6 AM rate (%)</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tokoli</td>
<td>142</td>
<td>10</td>
<td>6.6</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lokohoué</td>
<td>662</td>
<td>237</td>
<td>26.4</td>
<td>5.084</td>
<td>2.632–9.820</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; Odds ratio, 95% CI, and *P* value according to a Fisher exact test.
tropical storms and can drastically reduce the number of mosquitoes caught on humans. However, we never conducted mosquito collection when the weather was bad. Moreover, we observed that nocturnal temperatures were not different between rounds of collection or correlated with changes in biting behavior (see in the Supplementary Table 2 and 3). This suggests that local climatic conditions were unlikely to be responsible for the switch in *A. funestus* biting behavior during the study.

Here, we provide, to our knowledge, the first evidence for a substantial diurnal host-biting behavior of a major malaria vector in Africa. Indeed, during round 3 in Lokohoué, a large proportion of the aggressive fraction of *A. funestus* (26%) was collected after 6 AM. Of note, in both villages during round 1, the proportion of biting of *A. funestus* from 5 AM to 6 AM was >10%, suggesting that a diurnal biting activity was already present before the implementation of LLINs. The dogma that malaria vectors are strictly nocturnal may not be entirely true, especially if they have been exposed to intense selection pressure resulting from the scaling up of residual insecticide for malaria vector control. Moreover, in many studies in which the peaks of aggressiveness of *A. 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 behavior of malaria vector is worrying, because in rural Africa, villagers usually wake up before dawn to work in crops, and thus, 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 *A. funestus* is the dominant malaria vector [3, 28, 34]. Moreover, *A. 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 because of high nocturnal temperatures and low mosquito biting nuisances [20].

Of interest, increases in outdoor biting mosquitoes was observed in Tokoli, where the proportion of vector biting after 6 AM was the lowest. This contrasts with the situation in Lokohoué, where lower exophagy rates but higher late morning biting rates were observed. These findings raise crucial questions about the evolutionary processes involved in mosquito behavior in relation to 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 behavior of the host [31, 38]. Recent but massive selection pressure induced by vector control tools may have altered the human-vector interactions. Therefore, of note, one behavior among late biting and outdoor biting predominated in each village suggested that vector control interventions may select for different adaptive 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 despite insecticides might influence behavioral traits. Here, the *A. funestus* population was fully susceptible to deltamethrin, the insecticide used in Permanet 2.0. Thus, modifications of biting behavior observed after full coverage of LLINs cannot be attributed to pleiotropic effects or to the presence of any pyrethroid resistance mechanism. Adaptation of *A. funestus* to LLINs may result from a phenotypic plasticity or from selected behavioral traits. In Senegal and in Burkina Faso [39, 40], chromosomal forms of *A. funestus* were found to be associated with different resting, biting, or host preference behavior. We assume that, in southern Benin, a genetically distinct form of *A. funestus* might be selected by vector control interventions. Further investigations in cytogenetic, population genetics and mosquito behavior are, however, required to confirm this trend.

In conclusion, we found evidence for a modulation of *A. funestus* biting behavior after implementation of full coverage of LLINs at the community level. Vectors biting outdoor and/or at dawn when persons are not 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.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.
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