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## Insecticide resistance and behavioural adaptation as a response to long-lasting insecticidal net deployment in malaria vectors in the Cascades region of Burkina Faso

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The decline in malaria across Africa has been largely attributed to vector control using long-lasting insecticidal nets (LLINs). However, this intervention has prompted widespread insecticide resistance (IR) and been associated with changes in mosquito behaviour that reduce their contact with LLINs. The relative importance and rate at which IR and behavioural adaptations emerge are poorly understood. We conducted surveillance of mosquito behaviour and IR at 12 sites in Burkina Faso to assess the magnitude and temporal dynamics of insecticide, biting and resting behaviours in vectors in the 2-year period following mass LLIN distribution. Insecticide resistance was present in all vector populations and increased rapidly over the study period. In contrast, no longitudinal shifts in LLIN-avoidance behaviours (earlier or outdoor biting and resting) were detected. There was a moderate but statistically significant shift in vector species composition from *Anopheles coluzzii* to *Anopheles gambiae* which coincided with a reduction in the proportion of bites preventable by LLINs; possibly driven by between-species variation in behaviour. These findings indicate that adaptations based on insecticide resistance arise and intensify more rapidly than behavioural shifts within mosquito vectors. However, longitudinal shifts in mosquito vector species composition were evident within 2 years following a mass LLIN distribution. This ecological shift was characterized by a significant increase in the exophagic species (*An. gambiae*) and coincided with a predicted decline in the degree of protection expected from LLINs. Although human exposure fell through the study period due to reducing vector densities and infection rates, such ecological shifts in vector species along with insecticide resistance were likely to have eroded the efficacy of LLINs. While both adaptations impact malaria control, the rapid increase of the former indicates this strategy develops more quickly in response to selection from LLINs. However, interventions targeting both resistance strategies will be needed.

Long-lasting insecticidal nets (LLINs) and Indoor Residual Spraying (IRS) are the main malaria vector controls tools<sup>1,2</sup> recommended by the World Health Organisation (WHO)<sup>3</sup>. Both interventions have had a significant impact on malaria control in Africa; with LLINs being primarily responsible for the > 50% reduction in prevalence since 2000<sup>4</sup>. The success of these interventions rely on their ability to exploit innate aspects of malaria vector behaviour including a preference for feeding on humans (anthropophagy), inside houses (endophagy), during sleeping hours, and to rest inside houses (endophily) after feeding<sup>5,6</sup>. Consequently, LLINs and IRS are expected to work best against anthropophilic, endophilic and endophagic vectors that have high susceptibility

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to insecticides<sup>7,8</sup>. Historically, most of the major malaria vectors in Africa (members of the *Anopheles gambiae* complex) were described as having highly anthropophilic<sup>9,10</sup> and endophilic resting behaviour<sup>11</sup>, characterized by late night-biting<sup>11,12</sup>. This combination of traits is thought to account for the early success of current vector control approaches in Africa<sup>13</sup>.

However global progress on malaria control has stalled since 2015<sup>14</sup>, with a slowing of decline in cases and deaths, and even an increase in some settings. This stagnation has been most pronounced within a small group of high-burden countries including ten in Africa plus India. This slowdown is hypothesized to be driven by the widespread emergence of insecticide resistance (IR) in malaria vectors that has now been documented in almost all African countries<sup>15</sup>. Resistance has been facilitated by the reliance of almost all LLIN products on a single class of insecticides (pyrethroids). Vectors have developed a range of resistance mechanisms against pyrethroids including target site mutations<sup>16</sup>, cuticular resistance<sup>17</sup> and metabolic resistance<sup>18</sup>. Additionally, there are reports of vectors shifting key behaviours that predispose them to LLINs including their host choice<sup>19</sup>, biting time<sup>7,20</sup> and location (indoor versus outdoors<sup>21</sup>) and resting<sup>22</sup>. Such shifts can arise either through ecological changes in vector species composition (with more zoophilic, exophagic and exophilic species being favoured<sup>23,24</sup>) or evolutionary adaptations reflected by behavioural changes within species. Examples of within species behavioural changes include the apparent switch in biting time from late night to morning in *An. funestus* in Benin<sup>25</sup> and from indoor to outdoor resting in *An. arabiensis* in Tanzania<sup>19</sup>. Such ecological and adaptive changes have been documented in several settings<sup>26,27</sup>. Regardless of how changes in vector behaviour arise, they are expected to erode LLIN effectiveness; with outdoor biting alone estimated as being responsible for over 10 million malaria cases in Africa<sup>28</sup>.

Ecological shifts and evolutionary adaptations in mosquito behaviour could both reduce malaria control by enabling mosquitoes to avoid contact with LLINs. In contrast, IR allows mosquitoes to tolerate contact with LLINs by avoiding immediate mortality. Understanding the relative capacity of mosquito vectors to mount IR and behavioural avoidance in response to LLINs is necessary to estimate the risk posed by each process to malaria control and highlight where mitigation efforts should be prioritized. For instance, rapid emergence of behavioural avoidance mandates incorporation of interventions that target mosquitoes outside homes (e.g. spatial repellents, endectocides, larvicides etc.<sup>29</sup>). In contrast, the rapid emergence of insecticide resistance in vectors who continue to feed and rest indoors indicates that switching to alternative insecticide classes in indoor-based interventions (e.g. next generation nets) or using non-insecticidal methods inside the home (house screening<sup>30,31</sup>) may have greater impact.

Burkina Faso is amongst the 10 high burden malaria countries in Africa where prevalence appears to be rising despite several rounds of mass LLIN distribution<sup>32</sup>. The *Anopheles gambiae* complex (*An. arabiensis*, *An. coluzzii* and *An. gambiae*) and *An. funestus* species group are the primary vectors in this country<sup>33,34</sup>. Resistance to insecticides (DDT and Dieldrin) was first detected in Burkina Faso in the late 1960s<sup>35,36</sup>. Over the past two decades IR has intensified in vector populations in Burkina Faso<sup>15,37</sup> with levels now being amongst the highest in Africa<sup>38</sup>. This high rate of IR is hypothesized to be responsible for the limited impact of LLINs in Burkina Faso<sup>39</sup>, but less is known about the potential additional impacts of mosquito behavioural adaptations and changes in vector species composition. In the current study we estimated the potential epidemiological impact of temporal shifts in vector abundance, insecticide resistance and behaviour in two ways. First, we estimated expected human exposure to infected mosquitoes in term of the “Entomological Inoculation Rate” (“EIR”) between study years. The EIR is considered to be one of the most direct estimates of human exposure to malaria and has a relatively good correlation with human epidemiological outcomes such as malaria incidence<sup>40,41</sup>. Additionally, we estimated the degree of protection expected to be obtained from use of an effective LLIN during typical sleeping hours, as a function of the time and location of mosquito biting<sup>42</sup>. In combination this information will enable assessment of malaria transmission and the expected impact of current control tools as vector behavioural and physiological resistance rises.

In Burkina Faso, LLIN mass-distribution campaigns started in 2010. Approximately, ~8.4 and 10.5 million nets were distributed during campaigns held in 2010 and 2013 respectively. Much of the knowledge on malaria vector behaviours comes from before the period of mass LLIN distribution<sup>43–45</sup> and showed that > 50% of *An. gambiae* s.l. bite indoors and biting activity was concentrated late at night (01 h–06 h), with only few studies thereafter<sup>46,47</sup>. We conducted an observational study to measure the intensity and rate of change in insecticide and behavioural resistance traits within malaria vector populations over 2 years in the Cascades Region. The study was undertaken after a mass LLIN distribution held in July 2016 where ~10.6 million pyrethroid-only net were distributed. Aims were to (1) update information on vector ecology and behaviour in a high burden African country, (2) quantify and compare the magnitude and rate of change in insecticide and behavioural resistance traits, and malaria vector species composition and (3) estimate the potential impact of changes in these ecological and physiological traits on malaria transmission and LLIN effectiveness.

## Results

A total of 49,280 female mosquitoes were caught in Human Landing Catches (HLCs), of which most were female *An. gambiae* s.l. (N = 40,220; Table 1). A total of 927 mosquitoes were collected from the Resting Box Traps (RBTs) of which 584 were female *An. gambiae* s.l. (Table 2). Within the subset (~20% of total) of *An. gambiae* s.l. collected in HLCs that were identified to species level by polymerase chain reactions (PCR), 53.2% were *An. coluzzii*, 45.9% were *An. gambiae* and 0.28% were *An. arabiensis*. Within the 449 resting female *An. gambiae* s.l. identified by PCR, *An. coluzzii* was dominant (61.25%) followed by *An. gambiae* (38.08%), *An. arabiensis* (n = 2, 0.45%) and one hybrid between *An. coluzzii* and *An. gambiae* (0.22). Due to the low number of *An. arabiensis*, this species was not included in statistical analysis of species-specific differences.

Village	<i>An. coustani</i>		<i>An. funestus</i>		<i>An. gambiae s.l</i>		<i>An. nili</i>		<i>An. obscurus</i>		<i>An. pharoensis</i>		<i>An. rufipes</i>		<i>An. ziemanni</i>		Total
	IN	OUT	IN	OUT	IN	OUT	IN	OUT	IN	OUT	IN	OUT	IN	OUT	IN	OUT	
Dangouindougou	0	4	3	3	788	786	0	1	0	0	11	14	1	0	0	0	1611
Gouera	0	0	0	5	762	867	0	3	0	0	1	4	0	0	0	0	1642
Nianiagara	0	0	1	0	451	522	4	0	0	0	2	4	0	0	0	0	984
Nofesso	0	0	1	0	340	540	0	0	0	0	0	0	0	0	0	0	881
Ouangolodougou	0	0	0	0	265	409	0	0	0	0	1	0	0	0	0	0	675
Sitiena	31	12	1	1	2549	2589	160	306	5	8	25	34	0	0	1	0	5722
Tengrela	49	88	4	3	4579	4598	25	47	1	0	63	152	2	5	2	3	9621
Tiefora	1	2	4	2	3917	4754	14	24	0	0	52	119	0	0	0	0	8889
Timperba	0	1	0	1	445	413	0	1	0	0	0	0	0	0	0	0	861
Tondoura	0	0	1	0	1121	1096	2	5	0	0	1	1	0	0	0	0	2227
Toumousseni	0	2	2	2	2670	3056	8	17	0	0	14	29	0	0	0	0	5800
Yendere	2	1	0	4	1272	1431	5	9	0	0	9	9	0	0	0	0	2742
Total	83	110	17	21	19,159	21,061	218	413	6	8	179	366	3	5	3	3	41,655

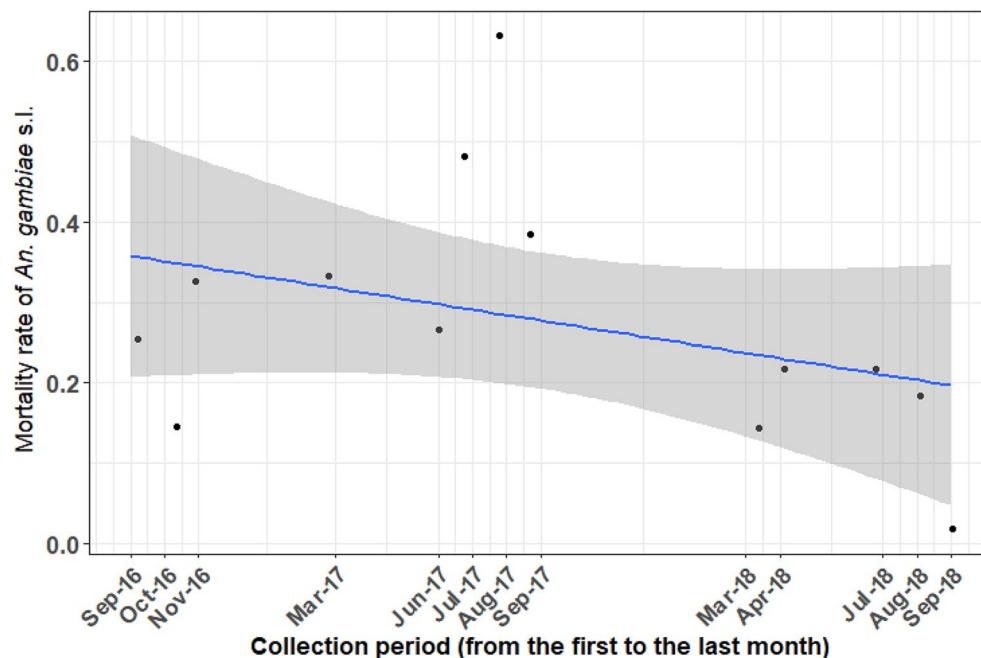
**Table 1.** Total number of female Anophelines caught using Human Landing Catches in 12 villages of southwestern Burkina Faso, from October 2016 to December 2018. Results are displayed by trapping location (IN = indoor and OUT = outdoor) and village.

	<i>An. coustani</i>		<i>An. funestus</i>		<i>An. gambiae s.l</i>		<i>An. nili</i>		<i>An. pharoensis</i>		<i>An. rufipes</i>		Total
	IN	OUT	IN	OUT	IN	OUT	IN	OUT	IN	OUT	IN	OUT	
Dangouindougou	0	0	0	1	2	4	0	0	0	0	0	1	8
Gouera	0	0	0	4	11	25	0	0	0	0	0	11	51
Nianiagara	0	0	0	0	8	6	0	0	0	0	0	0	14
Nofesso	0	0	0	0	1	2	0	0	0	0	0	0	3
Ouangolodougou	0	0	0	0	4	0	0	0	0	0	0	0	4
Sitiena	0	0	0	0	22	19	0	0	0	0	0	0	41
Tengrela	0	2	2	1	142	101	0	2	1	1	5	0	256
Tiefora	0	0	0	2	58	73	0	1	0	0	0	0	134
Timperba	0	0	0	0	2	1	0	0	0	0	0	0	3
Tondoura	0	0	0	0	12	11	0	0	0	0	0	0	23
Toumousseni	0	0	0	0	32	40	0	0	0	0	0	0	72
Yendere	0	0	0	0	6	2	0	0	0	0	0	0	8
Total	0	2	2	8	300	284	0	3	1	1	5	12	618

**Table 2.** Total number of female Anophelines caught using Resting Bucket Traps in 12 villages of southwestern Burkina Faso, from October 2016 to December 2018. Results are displayed by trapping location (IN = indoor and OUT = outdoor) and village.

**Insecticide resistance.** The overall mortality of *An. gambiae s.l.* in the 24 h following exposure to the diagnostic dose (DD) of deltamethrin was 23.33% [95% CI 14.63–32.05%], indicating a resistant population according to WHO criteria. The mortality of *An. gambiae s.l.* following exposure to the DD of deltamethrin declined significantly ( $df=1$ ,  $\chi^2=20.91$ ,  $p=0.001$ ; Supplementary Table 1) over the study period, falling from ~38% at the beginning (October 2016) to ~17% toward the end (September 2018, Fig. 1). As expected, mortality 24-h post-exposure generally rose with increased concentration of deltamethrin (0.25%: 64.14% [95% CI 55.27–73.0]; 0.5%: 86.03 [95% CI 81.87–90.2%]; 0.75%: 87.48% [95% CI 81.52–92.43%]). The predicted reduction in post-exposure mortality over time as observed with the DD was also detected at 15X the DD ( $df=1$ ,  $\chi^2=11.25$ ,  $p=0.001$ ; Supplementary Table 1). Long-term increases in IR were evident after controlling for statistically significant variation between villages (all concentrations) and season (only detected in bioassays using the DD; Supplementary Table 1).

**The human biting rate and *Anopheles gambiae s.l.* species composition.** Across the whole study period and pooling across sites, the mean Human Biting rate (HBR) of *An. gambiae s.l.* was ~17 bites per person per night (bppn). After controlling for seasonal and spatial variations, there was evidence of a 39% longitudinal reduction in HBR (Supplementary Table 2) from the beginning to end of the study period. Similarly, after controlling for variations due to season and village there was also evidence of 23% decline in the proportion of *An. coluzzii* relative to *An. gambiae* over the study period (Supplementary Table 2, Fig. 2).



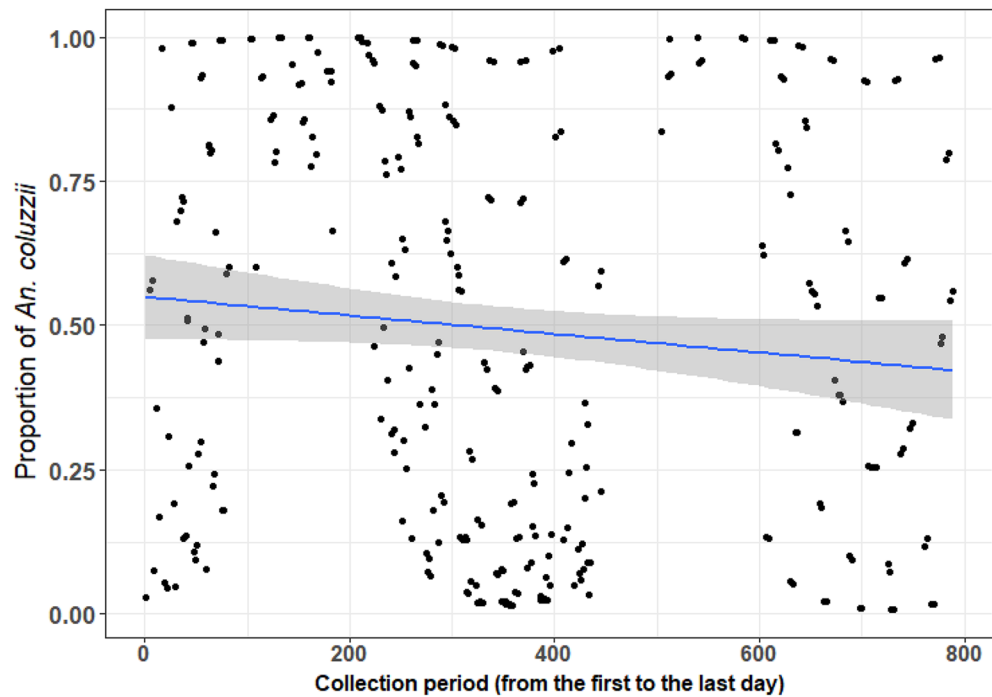
**Figure 1.** Mean predicted mortality of *Anopheles gambiae* s.l. 24 h following exposure to a diagnostic dose of deltamethrin across the study period. Black dots indicate mean predicted mortality at each study month between September 2016 to September 2018 across 9 villages in southwestern Burkina Faso. The blue line indicates the predicted linear change in *An. gambiae* s.l. across the study period based on the final model, with the grey-shaded area indicating the 95% confidence intervals.

***Anopheles gambiae* s.l. behaviours (biting and resting) and host choice.** *Anopheles gambiae* s.l. biting activity was at its lowest in the early evening (7–8 pm) and steadily increased to reach to a peak between 00 and 04 h; with the median biting time between 01 and 02 h. Overall, about 54% [95% CI ~51–57%] of *An. gambiae* complex members were collected host-seeking outdoors. However, there was no long-term change in the biting location across the study (Supplementary Table 2). Further analysis of the subset of *An. gambiae* s.l. that were individually identified to species level indicated that the proportion of outdoor biting varied between *An. gambiae* and *An. coluzzii* ( $df=1$ ,  $\chi^2=6.82$ ,  $p=0.009$ ). Specifically, *An. gambiae* was slightly more exophagic (% outdoor biting = 54.73%, [95% CI 52.35–57.12]) than *An. coluzzii* [% outdoor biting = 51.4%, 95% CI 48.9–53.9%]).

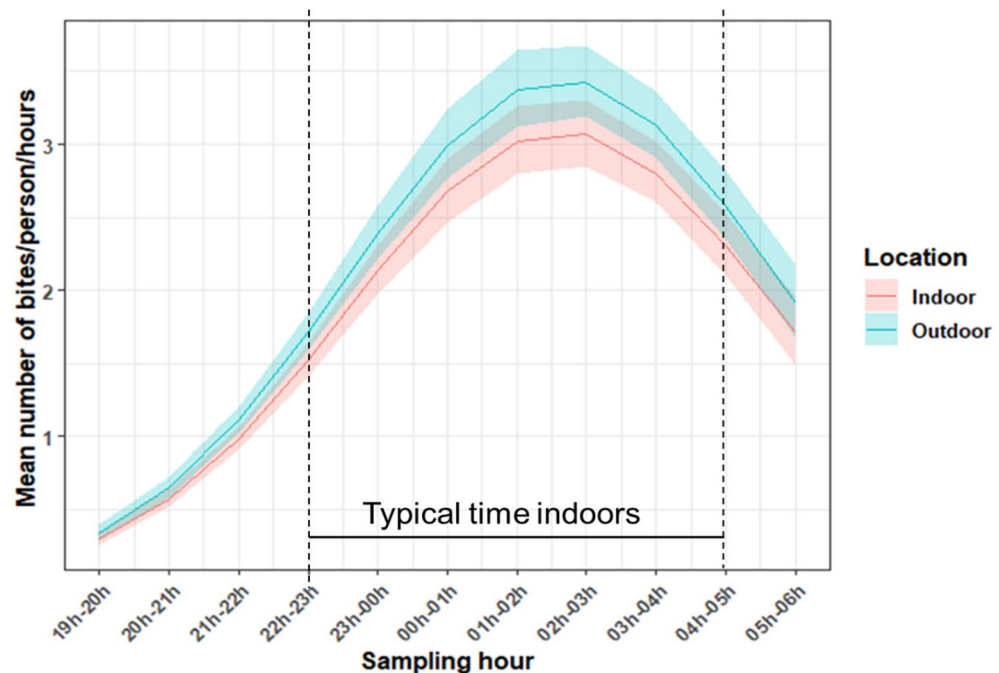
The timing of host-seeking activity was similar in the indoor and outdoor environment (Fig. 3). Although peak of biting occurred around midnight, some *An. gambiae* s.l. were still caught biting around 6 am (Fig. 3). No longitudinal change in median biting time was observed (Supplementary Table 2). Further analysis of the subsample of *An. gambiae* s.l. identified to species level indicated there was difference in the median biting time between *An. coluzzii* and *An. gambiae* (Supplementary Table 2). *Anopheles coluzzii* was found to bite earlier (11 pm–01 am) than *An. gambiae* (00 pm–02 am). The overall proportion of *An. gambiae* s.l. females resting outdoors was 46.97%. After controlling for the effect of season, there was no evidence of a longitudinal change in the proportion of female *An. gambiae* s.l. resting outdoors over the study period ( $df=1$ ,  $\chi^2=2.67$ ,  $p=0.32$ ). Of the 94 s.l. females from which blood-meals could be identified, 52% were from human only, 35.11% were cattle only, and 12.77% contained a mixture of cattle and human blood (Table 3); corresponding to an HBI of 64.9%.

**Malaria transmission and LLIN effectiveness.** Overall, 86.81% [95% CI 83.6–90.02%] of s.l. were caught biting between 10 pm and 5 am (corresponding to time when most people are indoors,  $P_{\text{in}}$ ). Furthermore, 85.45% [95% CI: 80.64–90.26%] of human exposure occurred when people are assumed to be indoors ( $\pi$ ). Thus, under the simplifying assumption that people remain under an LLIN between 10 pm and 5 am<sup>48</sup>, ~85% of exposure to *An. gambiae* s.l. could be preventable by LLIN use. Taking into account variations due to village and season, there was a modest but statistically significant decline (of ~7%) in the proportion of biting taking place when people are expected to be indoors (Supplementary Table 3,  $P_{\text{in}}$ :  $z=-3.14$ ,  $p=0.002$ , Fig. 4a) over the study period. Similarly, there was also ~10% decrease in the proportion of human exposure estimated to occur indoors (Supplementary Table 3,  $\pi$ :  $z=-3.72$ ,  $p=0.0002$ , Fig. 4b).

The mean sporozoite rate (SR) in *An. gambiae* s.l. across the study area was 3.48% [95% CI 1.51–5.26%]. After controlling for seasonal and spatial variations, there was evidence of a decline in mean SR ~5% to ~2% over the study period ( $z=-2.5$ ,  $p=0.01$ , Supplementary Table 4, Fig. 5). There was no significant difference in the SR between *An. coluzzii* and *An. gambiae*; or between *An. gambiae* s.l. captured host-seeking indoor versus outdoor (Supplementary Table 4). Annual Entomological Inoculation Rates (EIR) were calculated for the subset of 6



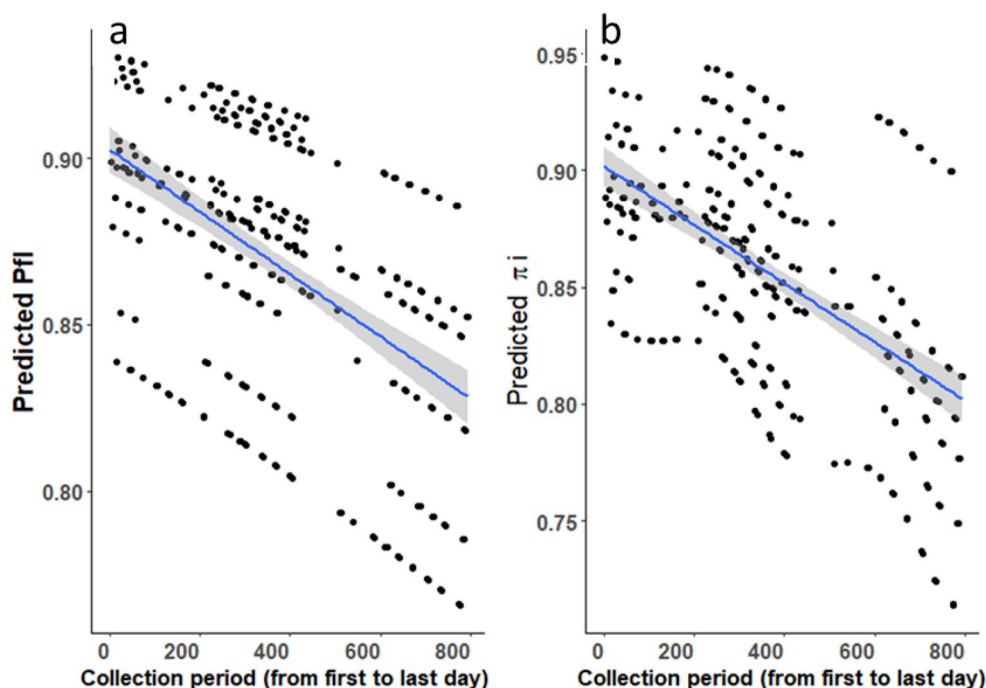
**Figure 2.** Mean predicted long-term trends in *Anopheles coluzzii* proportion within the *An. gambiae* complex from 12 villages in southwestern Burkina Faso. The blue line represents the predicted regression line from a model accounting for additional variation due to village and trapping location (inside and outdoors houses), with the grey-shaded area indicating the 95% confidence intervals.



**Figure 3.** Mean number of *An. gambiae* s.l. biting per hour (as assessed from Human Landing Catches) in 12 villages in southwestern Burkina Faso, from October 2016 to December 2018. Data are pooled over village and collection period. The period between the black vertical dashed lines indicates when most people are inside their dwelling and estimated to be sleeping under a nets. The blue and red curves indicate the predicted number of *An. gambiae* s.l. biting in outdoor and indoor settings, respectively; with the shaded areas indicating the 95% confidence intervals.

Species	Location	Cattle	Human	Human–cattle	Total	HBI
<i>An. arabiensis</i>	Indoor	1	0	0	1	0
	Outdoor	1	0	0	1	0
<i>An. coluzzii</i>	Indoor	18	23	4	45	0.6
	Outdoor	2	7	2	11	0.82
<i>An. gambiae</i>	Indoor	7	11	6	24	0.71
	Outdoor	4	8	0	12	0.67
Total		33	49	12	94	0.65

**Table 3.** Total numbers of blood-fed female *An. gambiae* s.l. caught using Resting Bucket Traps (RBT) in the 12 villages, from October 2016 to December 2018. Results are displayed by species, trapping location (Indoor or outdoors) and host blood source, pooled over village. ‘HBI’ indicate the ‘Human Blood Index’; estimated as the proportion of blood meals taken from humans out of the blood meals whose source could be identified. HBI indicate the proportion of mosquito that blood-fed on human.

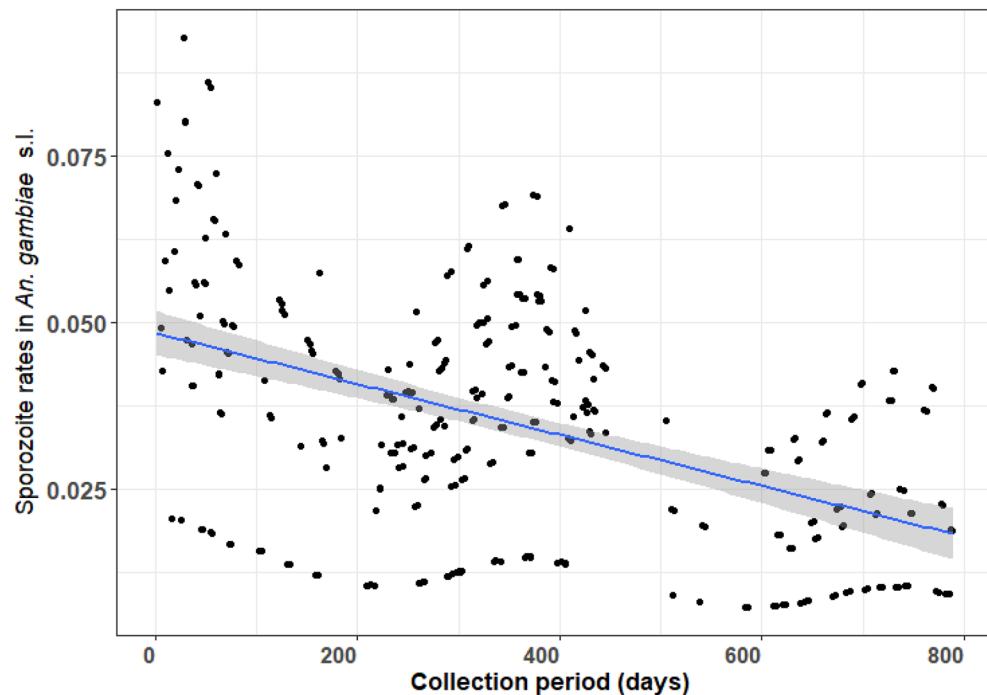


**Figure 4.** Predicted mean of (a)  $P_{fi}$  = the proportion of *An. gambiae* s.l. bites occurring when most people are inside their dwellings and likely asleep (e.g. between 10 pm and 5 am;  $P_{fi}$ ), (b)  $\pi_i$  = total human exposure to *An. gambiae* s.l. bites occurring indoors ( $\pi_i$ ) based on Human Landing Catch from 6 villages over 2 years (Oct 1st, 2016 to Dec 4th, 2018) in southwestern Burkina Faso. Dots represent the predicted values ( $P_{fi}$  and  $\pi_i$ ) at each sampling night. The blue lines represent the regression lines from the models and the grey-shaded area the 95% confidence intervals.

villages monitored in both year 1 (Oct 2016–Sept 2017) and year 2 (Oct 2017–Sept 2018). The annual EIR was considerably higher in year 1 (289.25 infective bites per person per year) compared to year 2 (94.81 infective bites per person per year). This decline was in accordance with the observed longitudinal declines in the human biting rate and SR across the study. Despite this decline, residents were still predicted to be exposed to ~29 infective bites per person per year after adjustment for the proportion of bites (~85%) that can be prevented through effective use of LLINs.

## Discussion

Here we tested for evidence of systematic changes in insecticide and behavioural resistance traits in malaria vectors over 2.5 years following a mass LLIN distribution in Burkina Faso. Consistent with expectation, there was a high prevalence of insecticide resistance (IR) within these malaria vector populations, which further intensified over the period of the study, with post-exposure mortality falling by ~50% (from 38% at the start to 17% at the end). In contrast, although baseline levels of “behavioural avoidance” traits were higher than expected (e.g. >50% outdoor biting and resting), behavioural phenotypes were relatively stable across the study period. There was however a reduction in *An. gambiae* s.l. abundance (Human Biting Rate) and a shift in vector species



**Figure 5.** Mean predicted longitudinal trend in sporozoite infection rates in *An. gambiae* s.l. (dots). Data are from *An. gambiae* s.l. collected in 12 villages in southwestern Burkina Faso using Human Landing Catches between October 2016 and December 2018. The blue line indicates the predicted temporal trend with the grey-shaded areas indicating the 95% confidence intervals.

composition across the study period; with a moderate reduction in the proportion of *An. coluzzii* relative to *An. gambiae*. The potential epidemiological impacts of these ecological changes are complex. On one hand, there was a significant drop in the Entomological Inoculation Rate between years, indicating a fall in human exposure and malaria transmission. On the other, the proportion of exposure to *An. gambiae* s.l. expected to be preventable by LLIN use also fell over the study period ( $\pi_i$ , ~7% fall); indicating a moderate erosion of protection from residual transmission through time. We hypothesize that the fall in  $\pi_i$  was due to a shift in vector species composition, with the slightly more exophagic *An. gambiae* increasing relative to *An. coluzzii* through time. Thus, we hypothesize that while both IR and avoidance behaviours are contributing to the limited impact of LLINs in this setting, behavioural traits appeared to be responding more slowly to selection from LLINs.

The rise in IR across this study is in line with similar increases observed over short time periods in other African settings<sup>49,50</sup>. This acceleration in IR appears to be a relatively recent phenomenon. For example, post-exposure mortality rates of *An. gambiae* s.l. to the DD of synthetic pyrethroids when first introduced in the 1970s (~95%<sup>51</sup>) was relatively similar to that in the 2010s<sup>52</sup>. The intensification of IR over the last decade is likely due to the initiation of mass LLIN distribution programmes in 2010. For example, post-exposure mortality of *An. gambiae* s.l. to the DD in one of our study sites (Tiefora) was ~39%<sup>53</sup> in 2014 compared to only ~15% reported here (2016–18). Additionally, in Tengrela (another study village), post exposure mortality to DD declined from ~92 to ~19% between 2011 and 2013<sup>37</sup>. In light of the rapid rise in IR, the government of Burkina Faso switched to new net products that combine pyrethroids with other insecticide classes in the most recent (2019) mass ITN distribution campaign (e.g. INTERCEPTOR G2: alpha-cypermethrin and chlorfenapyr; PBO-LLINs: deltamethrin and the synergist piperonyl butoxide).

While insecticide resistance increased significantly over the study, mosquito vector behaviours were generally static. However, some mosquito behavioural traits observed here differ substantially from historical reports in the study area; raising the possibility of more gradual long-term changes that may not be detectable over a few years. For example, previous studies from the Central, Plateau Central and West regions (2001–2015) of Burkina Faso reported *An. gambiae* s.l. was more likely to bite indoors than outdoors<sup>43,54</sup>, or had an even split between indoor and outdoor biting<sup>46</sup>. In contrast, here *An. gambiae* s.l. was more likely to host seek outdoors [~54%; 95% CI ~51–57%]. Additionally, a recent review of *An. gambiae* s.l. biting behaviour from a range of African countries concluded that in general >80% of vector bites occur indoors<sup>28</sup>, substantially higher than observed here. *Anopheles gambiae* s.l. were also found in indoor and outdoor resting traps with relatively similar frequency (53% versus 47%); in contrast to earlier studies in south western Burkina Faso where indoor resting was more common (~67%<sup>55</sup>). The Human Blood Index (~65%) of these *An. gambiae* s.l. populations was also lower than previously reported in Burkina Faso (>77%<sup>56</sup>) and Benin (>90%<sup>57</sup>). Thus, although there was no clear evidence of mosquito behavioural change across the two years of this study, comparison with historical and wider regional data suggest that slower acting behavioural adaptations may be occurring in tandem with more rapid rises in insecticide resistance.

LLINs have also been hypothesized to generate selection on mosquito biting times; with vectors shifting to bite earlier in the evening before people go to bed<sup>58</sup> to avoid this intervention. Here, *An. gambiae* s.l. still exhibited the characteristic “late night” pattern of biting, with no evidence of a shift over the study. The biting pattern observed here is consistent with early work on *An. gambiae* s.l. before mass LLIN use which also found biting rates peaked around 00 h<sup>12,59</sup>. Similarly, a study in western Kenya found *An. gambiae* s.l. and *An. funestus* continued biting late in the night even in the presence of LLINs<sup>60</sup>. However, other studies have detected substantial shifts in malaria vector biting times in association with interventions, including a recent report from Senegal of *An. funestus* biting earlier in the morning and even into daylight hours<sup>61</sup>. Adaptations in biting time may arise more rapidly in other settings due to variation in local ecology and background levels of IR.

Some previous studies that have reported shifts in malaria vector behaviour (e.g.<sup>27,62</sup>) did not identify *An. gambiae* s.l. to species level, thus preventing interpretation of whether changes were due to within-species adaptations or ecological shifts in species composition (e.g. reduction in the more endophagic *An. gambiae* relative to more exophagic *An. arabiensis*). While behavioural phenotypes remained relatively fixed within vector species in this study, there was evidence of a longitudinal shift in malaria vector species composition characterized by a reduction in *An. coluzzii* relative to *An. gambiae*. The proportion of outdoor biting was slightly higher in *An. gambiae* (55%) than *An. coluzzii* (51%), thus the relative decline in *An. coluzzii* is consistent with the prediction that LLINs have a greater impact on endophagic vector species. For example, highly anthropophilic and endophagic vector species like *An. gambiae*<sup>63</sup> and *An. quadriannulatus*<sup>64</sup> declined more substantially than the more zoophilic and exophagic *An. arabiensis* following ITN introduction in East Africa. This adds to the growing body of evidence from across Africa showing that LLINs can provoke shifts in malaria vector species communities. Such ecological shifts may occur more rapidly than longer-term behavioural adaptations within species.

Taken in combination, these results suggest that IR may be the first line of defence to LLINs compared to changes in mosquito behaviour, and/or that behavioural phenotypes have less capacity for adaptation than the traits underpinning IR. Differences in the rate of change in IR and mosquito behavioural traits also likely reflect their genetic basis. Mosquito behaviours are likely complex multigenic traits<sup>65</sup>, governed by many genes (some possibly antagonistic) that may limit capacity to respond quickly to selection. There is some evidence that behaviours like human host choice<sup>65</sup> and outdoor/indoor biting or resting<sup>66</sup> have a genetic basis in African malaria vectors. The genetic basis of IR traits is well established<sup>18,67</sup>, with some IR mechanisms linked to a single point mutation (e.g. Knock-down resistance<sup>16</sup>; indicating this type of resistance can respond rapidly and efficiently to selection. Vector populations in Burkina Faso have a high frequency of target site mutations (e.g. frequency of the L1014F type > 0.8<sup>68</sup>) which could have provided the foundation for quicker adaptation to insecticides in contrast to slower development of behavioural resistance.

The potential epidemiological impacts of the vector adaptations observed here are complex. On one hand, results indicate that use of effective LLINs during typical sleeping hours (10 pm–5 am) can still effectively prevent the bulk of human exposure to malaria vectors (~85%) in this setting. Additionally, vector abundance, sporozoite infection rates and the associated Entomological Inoculation Rate fell between the two years of this study; suggesting a transmission decline. However, this degree of protection expected from LLIN use (85%) is somewhat lower than estimated in other parts of Burkina Faso (90%, in 2002–2004), and Africa (95–99%<sup>69</sup>). Furthermore, the longitudinal changes in vector populations described here could further diminish LLIN impact in several ways. First, the intensification of IR is likely to erode the community protection afforded to non-net users. Second the proportion of exposure preventable by using LLINs ( $\pi_1$ ) was predicted to decline over the study period. Even if the proportion of human exposure preventable by LLIN use remained at current levels, high levels of residual transmission will persist because of the relatively high baseline abundance and malaria infection rates in these vector populations. People in this area are estimated to be exposed to between ~29 infective bites per person per year even if they sleep under an LLIN between 10 pm–5 am. The small shifts in  $P_{fl}$  and  $\pi_1$  observed through time could further increase this exposure. Interventions that tackle both insecticide resistant and outdoor biting mosquitoes will thus be needed to tackle residual transmission.

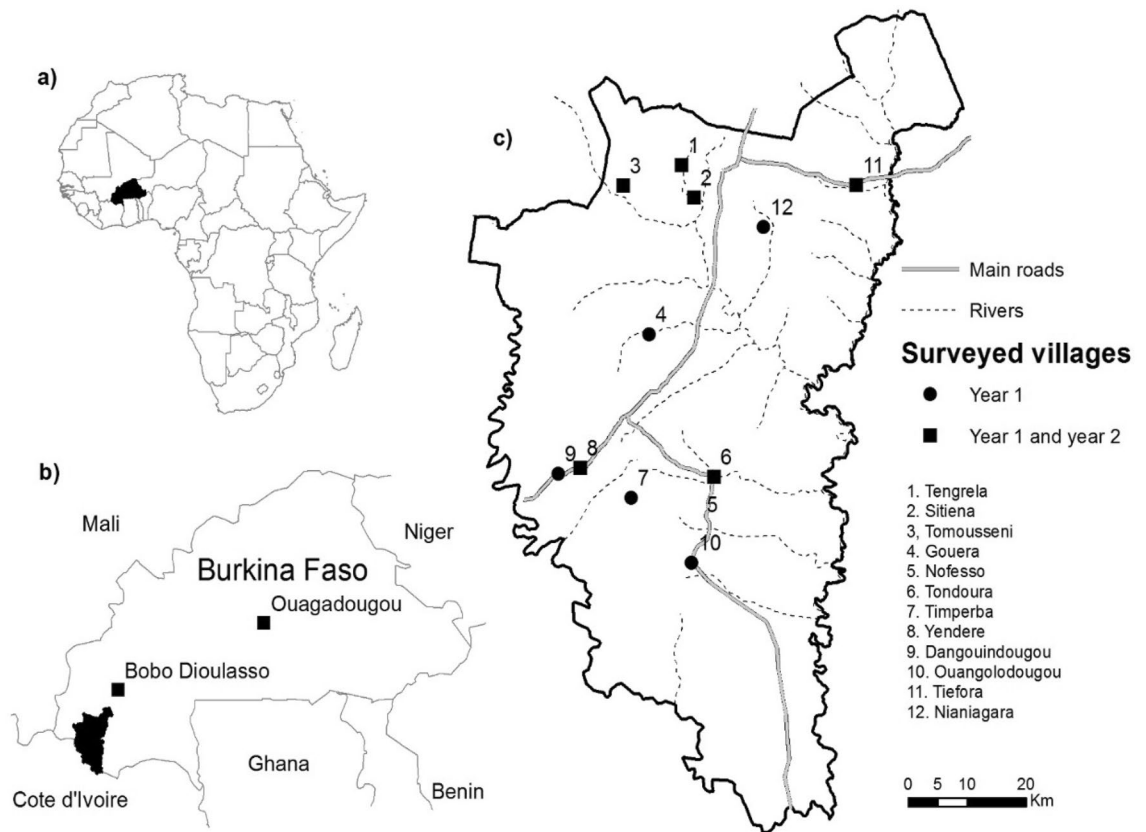
## Conclusions

In a longitudinal study in Burkina Faso, we show that IR increases more rapidly than behavioural adaptations following the mass distribution of insecticidal nets. Although mosquito behaviours stayed relatively stable over the study, baseline traits indicated a higher capacity for ‘behavioural avoidance’ of nets (through outdoor biting and resting) than historical data. This highlights the possibility that behavioural traits are adapting but at a more gradual rate than physiological resistance. As most human exposure to infected mosquitoes still occurs indoors in this and other African settings, effective indoor interventions should still be prioritized including the use of novel insecticide classes on nets, sprays and non-insecticidal approaches. However, the proportion of exposure occurring outdoors is notable and increasing. Supplementary measures tackling human exposure in and outdoors will thus be required to make further progress and tackle residual transmission in this and other high burden African settings.

## Methods

**Study site.** This study was conducted in 12 villages within the Banfora District, in the Cascades Region, south-western Burkina Faso (Supplementary Table 5, Fig. 6). In 2018, the population size of the region was 822,445<sup>70</sup>. The region has a humid savannah climate characterized by a rainy (May to October) and dry season (November to April). Annual rainfall ranged from 600 to 900 mm; with a mean temperature of ~26 °C [15.7 °C–38.84 °C] and humidity of ~62% [15.11–99.95%] during the study. Malaria is endemic in the region, with the incidence of *Plasmodium falciparum* being ~3 episodes per child (6–14 years old) during the six month annual transmission season<sup>32</sup>.





**Figure 6.** Map of the 12 study villages. (a) location of Burkina Faso within Africa, (b) study area in the Cascades Region in Burkina Faso, and (c) villages where mosquito collection took place. Circles represent the villages sampled for 18 months and squares represent the longer-term study sites where sampling was extended to 26 months. The map was generated using QGIS 3.16<sup>71</sup>. Background layers were downloaded for OpenStreetMap<sup>72</sup>, villages were digitalised by the authors using GPS coordinates collected in the field using a GARMIN eTREX 10 GPS.

**Insecticide resistance monitoring.** We aimed to measure IR in all 12 original study sites at least once during the wet and dry season of each year. Due to the limited availability of larval habitats in some villages and months (especially during the dry season), IR monitoring was only possible in 9 villages (Supplementary Tables 6 and 7). Larvae were collected from aquatic habitats and reared to adulthood under standard conditions<sup>73</sup>. Insecticide resistance assays were performed by exposing groups of 22–27 adult female *An. gambiae s.l.* to the World Health Organization discriminating dose for deltamethrin (DD = 0.05%, a concentration that can kill 99.9% of a susceptible population<sup>74</sup>), 5 times (0.25%), 10 times (0.5%) and 15 times (0.75%) the DD respectively (Supplementary Table 6). Mosquitoes were exposed to insecticide-treated papers obtained from the WHO/Vector Control Research Unit, University Sains Malaysia<sup>75</sup>, following WHO guidelines for Tube bioassays<sup>74</sup>.

**Assessment of human biting rate and behaviours.** Vector surveillance was conducted to measure human biting rates (HBR) and mosquito resting behaviours bi-monthly at all 12 sites for 16 months (Oct 2016–Feb 2018, Fig. 6). Additionally, surveillance continued for a further 10 months (Feb–Dec 2018) in a subset of 6 villages to generate longer-term data. The longer-term study villages were selected to achieve a relatively broad spatial distribution.

Host-seeking mosquitoes were sampled twice a month in each village from two different households per day using Human Landing Catches (HLC). Here, men collected mosquitoes landing on their exposed leg using a mouth aspirator and flash torches<sup>76</sup>. Collection took place both inside houses and outdoors (the peridomestic area) from 7 pm to 6 am each night. During each hour of collection, participants caught mosquitoes for 45 min, followed by a 15-min rest break. Participants involved in mosquito collections rotated between indoor and outdoor trapping stations each hour to avoid confounding location with individual differences in attractiveness to mosquitoes.

In addition, Resting Box Traps (RBTs) were placed in and outside houses<sup>77</sup> to sample resting mosquitoes. These RBTs were made locally using 20 L plastic buckets, with their inner surface covered with moistened black cotton cloth to create a high contrast and humid environment. On each night of collections, two RBTs were placed in the same households where HLCs took place (set within different houses in the compound). Inside houses, RBTs were placed on the floor in a relatively shaded corner of the sitting room. Two RBTs were also set

outdoors at ~ 8 m from houses to capture outdoor resting mosquitoes. RBTs were set up at approximately 7 pm and emptied the following morning (~ 5 am) using electrical aspirators.

**Mosquito processing.** Mosquitoes collected in HLCs and RBTs were transferred to an insectary in Banfora town and sorted to species complex level using morphological keys<sup>78</sup>. Of those identified as belonging to the malaria vector group *Anopheles gambiae* s.l., a subset of 7852 females (~ 20% of total) were selected to provide a representative sample from each month, village, trapping location (indoor vs outdoor) for identification to species level by PCR<sup>79</sup>. The subsampling strategy is described elsewhere<sup>80</sup>. Furthermore, malaria infection in mosquitoes was assessed by testing for the presence of *Plasmodium falciparum* circumsporozoite protein (CSP) in their head and thoraxes using a monoclonal sandwich Enzyme Linkage Immuno-Sorbent Assay (ELISA) developed by<sup>81</sup>. Additionally, female *An. gambiae* s.l. from the RBT collections were visually graded according to their repletion status (abdominal condition) into categories of blood-fed, unfed, gravid, and half gravid<sup>82</sup>. Blood-meal identification was carried out on blood-fed *An. gambiae* s.l. to determine the origin of their blood-meal (human, cattle or both<sup>83</sup>) using a direct Enzyme Linkage Immuno-Sorbent Assay (ELISA<sup>84</sup>). The degree of “anthrophagy” in vectors was estimated in terms of the “human blood index” (HBI)<sup>85</sup>, defined as the proportion of identified blood meals taken from humans. In ELISA tests (for sporozoite and blood meal source identification), two technical replicates of each sample were run in two different microplates at the same time and retested in cases where the first result was ambiguous. The absorbance of the solutions/reactions at the end of each ELISA was measured using microplate reader (ELX808; BIO-TEK) at 450 nm. To avoid any false positives (due to background noise), a sample was considered positive for an assay when its optical density (OD) was twofold higher than the average of the OD of both negative controls. Positive controls were also used in all ELISAs to ensure the procedure was working.

**Data analysis.** Statistical analysis was conducted with the aim of testing for “longitudinal shifts” (over 26 months) in key indicators of vector demography, species composition, insecticide resistance (IR) and behaviour using Generalized Additive Mixed-Models (GAMMs) in the R statistical software (version 3.6.1 of 2019-07-05)<sup>86</sup>. Long-term trends in all ecological, behavioural and insecticide resistance variables were assessed after controlling for spatial (between-village) and environmental (season, temperature and humidity) variations. The key response variables were: (i) the proportion of mosquitoes dying within 24-h post-exposure to 1X DD, 5X DD and 10X DD, (ii) human biting rate (HBR) and species composition (proportion of *An. coluzzii* in *An. gambiae* s.l.), (iii) proportion of outdoor biting and resting, (iv) median biting time, (v) proportion of human exposure to bites, (vi) the sporozoite rate and (vii) the entomological inoculation rates.

A model was constructed for each response variable (Supplementary Table 8) to assess longer-term trends and seasonality occurring over the 26 months of the study. For the longer-term trend, a temporal variable was created by assigning a continuous value to each day of collections from the first (1 October 2016) until the last (4 December 2018), hereafter called “Longer\_term” (Supplementary Table 8). This was incorporated into models as a continuous covariate to test for evidence of a consistent temporal rise or decline after accounting for seasonal and spatial variation. Seasonality was incorporated by fitting a smoothing spline where each day of the year was classified on a scale running from 1 (set as January 1st) to 365 (December 31st) hereafter called “Season” (Supplementary Table 8). Spatial variation was also assessed by fitting village as a fixed effect. The mean nightly temperature and humidity at collection households were derived from hourly values (7 pm–6 am) obtained from data loggers and included as additional explanatory variables (Supplementary Table 8).

However, due to the low number of mosquitoes caught in RBTs, it was not possible to build a model for testing for spatial and long-term variation in the abundance of resting mosquitoes. Mosquito host choice was assessed in terms of the Human Blood Index (HBI) defined as the proportion of blood-fed *An. gambiae* s.l. that tested positive for human blood out of the total from which blood-meals were identified (N = 94). We also calculated the Entomological Inoculation Rate (EIR); defined as the average number of infective bites a person (ibppn) would expect to receive from *An. gambiae* s.l. in a given location per year for the six villages that were monitored over 2 years. Here EIR was calculated as the product of the mean nightly human biting rate (*ma*) and vector sporozoite infection rates (SR) multiplied by 365 days<sup>41</sup> monitored over two years.

**Mosquito mortality rate after exposure to insecticide.** Insecticide resistance was measured in terms of mosquito mortality 24 h after exposure to deltamethrin (different doses). According to World Health Organisation (WHO) guidelines<sup>74</sup>, for a test to be validated the mortality should be less than 5% in the control groups. If, mortality in the control group ranges between 5 and 20%, the guidelines recommend using the Abbott formula<sup>74</sup> to correct for anticipated background mortality. If the mortality rate in the control group exceeds 20%, results from the test should be discarded. Here, average mortality rates recorded in the control group on the same day as insecticide exposures were performed were all below 5%, and could thus be retained for use without correction. We constructed separate models of mosquito mortality for each of the insecticide concentrations used (Models 1 to 4, Supplementary Table 8). As the response variable is all these analyses was binary (e.g. ‘0’ is alive, ‘1’ is dead); all models used a binomial distribution. Each model tested for seasonal and long-term variation in mosquito mortality following insecticide exposure (Supplementary Table 8), and variation between villages.

***Anopheles gambiae* s.l. species composition, biting and resting behaviours.** Models were constructed to test for longer-term (systematic increase or decrease across the 26 month study period) and seasonal variation in species composition (Model 5), HBR (Model 6) and outdoor biting (Model 7 and 8) and resting (Model 12) as described in Supplementary Table 8. Additionally, long-term and seasonal variation in the mean mosquito biting times (both *An. gambiae* s.l. overall, and by species; Model 10–11). Model 6 (Supplementary Table 8), for the

HBR included an interaction term between village and year in addition to other main effects. Model for mosquito median biting time includes an interaction between village and location. Vector species composition was defined as the proportion of *An. coluzzii* within *An. gambiae* complex. This was defined as binary proportion as only two major vector species (*An. coluzzii* and *An. gambiae*) account for almost all within the *An. gambiae* complex in this region. The proportion of outdoor biting was defined as the proportion of *An. gambiae* s.l. collected in outdoor HLCs out of the total in outdoor and indoor HLCs. As described in Supplementary Table 8, different distributions were incorporated into models depending on the nature of the response variable (e.g count data fit to Poisson or Negative Binomial model; proportion data fit to a binomial distribution).

**Human exposure to *Anopheles gambiae* s.l. bites.** Data on the time and location of biting were combined for estimating two metrics of human exposure to bites from *An. gambiae* s.l.: the proportion of *An. gambiae* s.l. caught during hours when typically,  $\geq 50\%$  of people were indoors and likely to be in bed ( $P_{in}$ ). Based on visual surveys described in<sup>80</sup> over 672 households, we estimated that  $> 50\%$  of residents were indoors between 10 pm and 5 am consistently across the study period; although more recent, detailed anthropological investigation indicates time indoors may vary substantially between individuals and seasons and be lower assumed here<sup>48</sup>. The  $P_{in}$  (Model 14 in Supplementary Table 8) was estimated following<sup>42</sup> by dividing the total number of s.l. collected indoors and outdoors when  $\geq 50\%$  of people were indoors (10 pm to 5 am) by the total number collected that night. The proportion of human exposure to bites that occur indoors ( $\pi_i$ ) was then calculated by dividing the number of *An. gambiae* s.l. collected indoors between 10 pm and 5 am by itself plus those collected outdoors when  $> 50\%$  of residents were outdoors and thus unprotected by LLINs (between 7–10 pm and 5–6 am (Model 15, Supplementary Table 8)). As  $\pi_i$  and  $P_{in}$  are proportions, they were modelled following a binomial distribution.

Within the R statistical software<sup>86</sup> GAMMs within the ‘mgcv package’<sup>87</sup> augmented with the lme4 package<sup>88</sup> known as GAMM4 were used to test for associations between IR, all vector ecological and behavioural metrics and explanatory variables of village, season, “longer-term” trend and environmental factors. Details of fixed and random effects and distribution are given in Supplementary Table 8.

**Ethical approval.** Ethical clearance was obtained from the Ethical Committee for research in Health of the Ministry of Health of Burkina Faso (EC V3.0\_CERS N°2016-09-097) and the Institutional Bioethical Committee of the local research institution (National Malaria Research and Training Centre, CNRFP) under EC V3.0\_N°2016-026/MS/SG/CNRFP/CIB) and the Liverpool School of Tropical Medicine (Certificate 16-038). Prior to starting the research, the project aims, and objectives were explained to community leaders in each village. Signed informed consent was also obtained from all household owners where mosquitoes were collected, and volunteers who took part in mosquito collections by HLC. We confirm that all methods were performed in accordance with the relevant guidelines and regulations.

## Data availability

All data are provided as Figures and Supplementary materials.

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## Author contributions

A.S. designed the study, conducted the field data collection, did the molecular analysis, performed the data analysis, interpreted the results, and drafted the manuscript. W.M.G., N.F.S., H.R. and H.M.F. contributed to the design of the study and the sampling protocol and provided comments upon the manuscript. H.M.F. was a major contributor in writing this manuscript. M.T., F.C. and P.O. contributed to the molecular analysis in the labs. L.N., J.M., contributed to the data analysis. All authors read and approved the final version of the manuscript.

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## Competing interests

The authors declare no competing interests.

### Additional information

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