Isotopic evidence that aestivation allows malaria mosquitoes to persist through the dry season in the Sahel

Roy Faiman1,3,4, Alpha S. Yaro2,3, Adama Dao4,5, Zana L. Sanogo2, Moussa Diallo2, Djibril Samake1, Ousmane Yossi2, Laura M. Veru1, Leland C. Graber1, Abigail R. Conte1, Cedric Kouam1, Benjamin J. Krajacich1 and Tovi Lehmann1,4

Data suggest that the malaria vector mosquito Anopheles coluzzii persists during the dry season in the Sahel through a dormancy mechanism known as aestivation; however, the contribution of aestivation compared with alternative strategies such as migration is unknown. Here we marked larval Anopheles mosquitoes in two Sahelian villages in Mali using deuterium (2H) to assess the contribution of aestivation to persistence of mosquitoes through the seven-month dry season. After an initial enrichment period, 33% of An. coluzzii mosquitoes were strongly marked. Seven months following enrichment, multiple analysis methods supported the ongoing presence of marked mosquitoes, compatible with the prediction that the fraction of marked mosquitoes should remain stable throughout the dry season if local aestivation is occurring. The results suggest that aestivation is a major persistence mechanism of An. coluzzii in the Sahel, contributing at least 20% of the adults at the onset of rains. This persistence strategy could influence mosquito control and malaria elimination campaigns.

Africa continues to carry a disproportionately high share of the global malaria burden, amounting to 94% of all malaria cases and deaths. Recent evidence suggests that Anopheles coluzzii persists in the West African Sahel as aestivating adults, that is, in a state of summer dormancy typically characterized by suppressed reproduction and/or growth that facilitates extended survival during harsh conditions1–5, supporting previous studies with similar assertions6–8. Although these studies have demonstrated that aestivation does occur, they do not rule out additional strategies and have not assessed the importance of any strategy in terms of its relative contribution to the persistence of mosquitoes over the dry season. In the West African Sahel, malaria transmission starts following the monsoon rains, and cases peak in the late wet season, between September and November, when mosquito breeding sites are abundant. During the ensuing seven-month dry season (December–May/June typically) rains cease completely, and surface water dries up shortly after, preventing mosquito reproduction. Mosquito populations plummet, and malaria transmission diminishes consequently9,10. Survival of Anopheles gambiae s.l., the primary malaria vector during the wet months, averages 3–6 weeks typically11,12, suggesting no mosquitoes should survive the six- to eight-month dry season. Yet during this period, An. coluzzii (formerly An. gambiae M form) adults can be found at a very low frequency, while An. gambiae sensu stricto (hereafter, A. gambiae) and An. arabiensis are virtually absent1,4,13. Days after the first rain, but noticeably before a new generation of mosquitoes can develop to adulthood, An. coluzzii reappear1,4,13 (Fig. 1a). The ‘dry-season malaria paradox’ of dry-season mosquito persistence has been puzzling malariologists and medical entomologists for almost a century14–16.
To date, direct evidence such as the capture of aestivating adult mosquitoes has remained limited\(^2\). Interestingly, for a brief period in late March to early April, An. coluzzii densities rebound inexplicably\(^1\). The source of the mosquitoes during this period has been attributed to either local aestivation or long-distance migration. During this abrupt period of the dry season (late dry-season peak), mosquito density spikes to levels often superseding those of the wet season, only to vanish again until the first rain, 6–10 weeks later\(^1\) (Fig. 1).

Tracking insects over an extended time remains a challenge given their small size and large dilution in the unmarked population\(^2\). Optimal marking should not affect the behaviour, development, longevity or reproduction of mosquitoes, should be simple to apply, should persist throughout the duration of the experiment, and should not be prohibitively priced to apply and detect\(^3\). Stable isotopes (for example, \(^{13}\)C and \(^{15}\)N most commonly) have previously been used to mark and track several insect species, including mosquitoes on a limited spatio-temporal scale\(^2\) (Supplementary Information). With likely long-term residual effects of carbon and nitrogen through assimilation into the local food webs as basic nutrients and metabolites\(^4\), and without evaporation, we selected hydrogen (in the form of deuterium oxide, \(^2\)H\(_2\)O), which behaves like water (is non-residual) and is expected to vanish after the dry season\(^5\). In addition, adding substantial amounts of sources of carbon and nitrogen, which are limiting factors in such ecosystems, might change suitability of the larval sites for mosquitoes and other organisms, which we wanted to avoid\(^6\). The method of stable isotope enrichment has clear advantages, but issues such as cost and future marking studies in recurring locations should be considered when selecting an isotope\(^7\). Although not tested on mosquitoes in the laboratory for periods beyond 50 days (An. gambiae s.l. maximum survival in insectary conditions), \(^2\)H (deuterium) marking potentially enables long-term mosquito marking, albeit with an expected signal degradation over a prolonged period as was evident in a previous study on mosquitoes, which provided valuable data for modelling the marking degradation over a seven-month period\(^8\). Hydrogen loss from chitinous tissues caused by deuterium exchange with protium (\(^1\)H) and enzymatic digestion and hydrolysis was shown to occur in regions of \(\alpha\)-chitin of the cuticle of insects and crustaceans\(^9\). The long-term persistence of \(^2\)H in mosquitoes seems to depend on the initial enrichment level as well as the tissue it enters; \(^2\)H in chitin of inert butterfly scales or keratin of avian feathers, for example, seems to behave differently from chitin of living mosquito cuticle, as exhibited by \(^2\)H degradation through exchange. Despite degradation of the marking over extended periods, \(^2\)H levels in mosquitoes remain above natural levels for an extended duration after experimental enrichment\(^1\), and an even higher experimental dose may outlast the aestivation period.

In this Article, we aimed to empirically estimate the contribution of aestivation to the persistence of mosquitoes through the dry season, assessing the fraction of aestivators that survive to establish the next generation of mosquitoes in the following wet season. This was achieved by marking a fraction of the adult population through natural larval-site \(^2\)H enrichment by the end of the wet season and evaluating the changes in the fraction of marked mosquitoes through

---

**Fig. 1** | Mark–release–recapture experiment timeline. **a**, Stable isotope mark–release–recapture timeline. Line graph (black) is a schematic following Sahelian An. coluzzii density by month (after ref. \(^2\)). Wet season (defined by availability of surface waters) is depicted in light-green background, the dry season in light orange. Larval sites were enriched with \(^2\)H during the end of the 2017 wet season (horizontal grey arrow) and until all water dried up in late November (dark orange arrow). Indoor mosquito sampling times throughout the 2017 wet season (horizontal grey arrow) and until all water dried up in late November (dark orange arrow). **b, c**, Mosquito \(^2\)H-enrichment distributions schematic contrasting background pre-enrichment (blue) with approximately one to two months after enrichment (b) and five to seven months (T1–T3) after enrichment during the late dry season and onset of rains (c). The 75th quantile of the background populations (starting from the 3rd quartile (Q3)) is the grey area depicting the upper tail (top 25%), showing no overlap between the background (blue) and enriched (red) mosquito populations early in the study. Because of the expected degradation in \(^2\)H over three to eight months after marking, we hypothesized that the gap between the means of the natural and the enriched populations will contract, and some marked mosquitoes whose \(^2\)H values have degraded enough may overlap with others in the upper tail of the background population, forming an excess of mosquitoes in that part of the distribution. Accordingly, the proportion of mosquitoes in the post-enrichment population whose \(^2\)H values were above the 3rd quartile (within the 75th quantile) of the pre-enrichment distribution would be larger than the expected 25%. Box-and-whisker plots (bottom) show the first, second (median) and third quartiles (box), and the whiskers extend to the highest observation up to 1.5 times the inter-quartile range (IQR = Q3 – Q1).
the dry season, the late dry-season peak and especially at the onset of the rain of the subsequent wet season, before a new generation of mosquitoes could have emerged (Fig. 1a). If aestivation is the only way An. coluzzii persists, the fraction of marked mosquitoes should remain stable. Finding no marked mosquitoes or a much smaller fraction of marked mosquitoes would be evidence against local aestivation or for low contribution of aestivation, respectively, thus supporting alternative strategies.

**Enrichment phase: variation between villages and species**

Isotopic enrichment of natural larval sites began in late September when all three anopheline vector species were present and ended when all surface waters dried up in late November (Fig. 1a). To determine the magnitude of adult enrichment and assess the proportion of marked An. coluzzii at the time, a comparison of the species composition and their respective enrichment level was done in the focal villages. Species compositions in Thierola and M'Piabougou before and during enrichment (September–November) were similar (September: exact test \( P > 0.46 \); November: exact test \( P > 0.46 \); overall using Cochran–Mantel–Haenszel statistics: Non-zero correlation \( > 0.99; P > 0.46 \); November: exact test \( P > 0.46 \)).

The first half of the dry season (December–February) is characterised by low contribution of aestivation, respectively, thus supporting the hypothesis that certain marked mosquitoes whose \(^3\)H values had degraded enough may ‘accumulate’ in the upper tail of the population’s \(^3\)H distribution, thus inflating the expected percentile. Accordingly, the proportion of mosquitoes in the post-enrichment population whose \(^3\)H values were above the third quartile of the pre-enrichment distribution would be larger than the expected 25% (Methods). After the last larval site dried up in Thierola and in M’Piabougou (Methods), no rain fell in the area during the dry season and no surface water was available for larvae to develop in. Therefore, any mosquito with an \(^3\)H value above natural levels was considered an aestivator marked during the 2017 wet season. Due to the low numbers of mosquitoes in the villages during December–February, only seven were collected (six from Thierola; all were An. coluzzii. As the late dry-season peak unfolded in March–April\(^1\), an additional 102 mosquitoes were collected from Thierola, all An. coluzzii (\( n = 100; 2 \) failed to produce a PCR band), in accord with previous results on seasonal species composition\(^12\).

During the early dry season (December–February), one to three months after marking, the \(^3\)H values of mosquitoes were lower than those during the enrichment period (October–November), but two of the seven mosquitoes exceeded the highest \(^3\)H values measured in the pre-enrichment levels (142 ppm; Fig. 3a), indicating they were marked mosquitoes. Therefore, approximately one-third of the mosquitoes in December–February from Thierola (Fig. 3a; \( ^{3}\)H > 152 ppm, one from mid-December and another from the end of January) were considered aestivators, or 29% from the focal villages (two of seven mosquitoes).

During March–April, 24% of the mosquitoes collected in Thierola exceeded the highest \(^3\)H value of the pre-enrichment period (142 ppm; Fig. 3a, b), and -10% surpassed 145 ppm. As predicted, the proportion of mosquitoes above the third quartile of the pre-enrichment distribution was 58%, indicating an excess of 33%. More conservatively, the proportion of mosquitoes in the post-enrichment distribution that had values above the 95% upper confidence limit (UCL) of the third quartile (of the pre-enrichment distribution) was 43%, indicating a significant excess of at least 18% (Fig. 3b).

The shrinking gap between the pre- and post-enrichment values raised the possibility that seasonal variation in natural \(^3\)H might confound the results. Therefore, we compared the post-enrichment distribution in March–April 2018 (late dry-season peak) with the same period in 2016 (Fig. 3c). Because only females were available from the 2016 collections, we excluded males from the 2018 sample. Notably, the \(^3\)H distribution of March–April 2016 had higher median and maximum values (137.2 ppm; Fig. 2b, c) than the corresponding values of the pre-enrichment distribution of September 2017 (134.3 ppm) and even the post-enrichment distribution of March–April 2018 (136.9 ppm; Fig. 3c), suggesting a seasonal and/or inter-annual variation in natural \(^3\)H. Likewise, a lower proportion of post-enrichment mosquitoes exceeded the maximum of the 2016 distribution (6.5%; Fig. 3c), and no evidence was found for an excess of post-enrichment mosquitoes above the 2016 third quartile (~2%; Fig. 3c).

We used the finite mixed-distribution model (FMM; Methods) as a reference-free approach to evaluate the presence and composition of the March–April population. The result for females (\( n = 62 \)) indicated the mixture of two subpopulations, with the larger subpopulation (81%) having a mean of 136.3 ppm \(( P < 0.001)\) and variance of 5.7 ppm\(^2\), and the smaller subpopulation (19%) having a mean of 144.2 ppm \(( P < 0.001)\) and variance of 4.5 ppm\(^2\), which were statistically separate \(( P < 0.0033; \text{Fig. 3d})\).

**Tracking marked mosquitoes during the onset of rain**

The first rain falls typically between mid-May and mid-June, and the first larvae were spotted in the newly formed larval sites 10–20 days after the first rain\(^13\). The first rain of 2018 fell on 24 June (see timeline, Fig. 1).

---

[Full citation and link for the article provided]
Hence, mosquitoes collected from 15 May to 5 July represent adults that in high probability were not mixed with the young adults that developed in the newly formed larval sites. Although a few putative aestivators may remain for a couple of additional weeks, their proportion is expected to decline rapidly. Accordingly, we assumed that mosquitoes collected from 17 July to 30 July represented mostly the new cohort of adults that had developed in the freshly formed larval sites. All mosquitoes tested from M’Piabougou were An. coluzzii (100%, n = 107), as were 99% (n = 186) from Thierola, in agreement with previous results1,4,13,31. The first A. arabiensis and A. gambiae were detected in Thierola on 22 June and 5 July, respectively.

Although lower than during the late dry-season peak (March–April), the 2H values of mosquitoes from Thierola and M’Piabougou at the onset of rains (15 May–5 July) were higher than those of the pre-enrichment period (September 2017), and their right tail exceeded the highest 2H values measured in the pre-enrichment population (Fig. 4a). As expected on the basis of the aestivation hypothesis, three weeks later, the 2H median and third quartile dropped in both villages (median difference of 1.6 ppm, P < 0.0023, t1 = 3.1; third-quartile difference of 2.2 ppm, P < 0.0001, t1 = 4.0; 0.9 quantile difference of 3.4 ppm, P < 0.022, t1 = 2.3, quantile regression), representing the first generation of mosquitoes that developed in the newly formed larval sites (late July) (t1 denotes the t statistic with 1 df). Notably, the difference increased at higher quantiles (P < 0.052, χ2 = 5.94), indicating that the greatest difference was related to highest 2H values. As expected assuming stable natural 2H levels, the 2H range of this first cohort of the 2018 wet
season was similar to that of the pre-enrichment period (September 2017, median difference of 0.5 ppm, $P > 0.53, t_1 = 0.7$; third-quartile difference of 0.4 ppm, $P > 0.4, t_1 = 0.5$; 0.9 quantile difference of −1.5 ppm, $P > 0.09, t_1 = −1.7$, quantile regression). The difference between Thierola and M’Piabougou was minimal and non-significant (median difference of 0.42 ppm, $P > 0.31, t_1 = 1.0$; third-quartile difference of 0.6 ppm, $P > 0.5, t_1 = 1.2$; 0.9 quantile difference of 0.75 ppm, $P > 0.4, t_1 = 0.8$, quantile regression); thus, we pooled mosquitoes from these villages at this time point. In May–July, 6.5% of the population have exceeded the highest value in September (142.2 ppm; Fig. 3a,b; four mosquitoes exceeded the 145 ppm mark).

Assessment of excess mosquitoes whose $^2$H values were above the third quartile of the pre-enrichment population (Methods) revealed 45.5% of post-enrichment mosquitoes, indicating an excess of 21%. Moreover, 3.4% of the post-enrichment mosquitoes represented excess over the 95% UCL of the third quartile of the pre-enrichment distribution, that is, a statistically significant excess (Methods). To further evaluate the presence of enriched mosquitoes in the focal villages Thierola (females only) using box and whisker plots (bottom) and histogram (top) and employing the quartile method. d. The composition of the post-enrichment females ($n = 62$) using the FMM assuming two subpopulations (initial values of mean and variance 136 ppm and 8 ppm$^2$ for subpopulation 1 and 144 ppm and 5 ppm$^2$ for subpopulation 2 (Methods)). The results support a mixed population with a mean of 136.3 ppm and variance of 5.7 ppm$^2$ ($P < 0.001, 81$%) and a smaller subpopulation with a mean of 144.2 ppm and variance of 4.5 ppm$^2$ ($P < 0.001, 19$%), which are significantly separated ($P < 0.0033$). Box-and-whisker plots show the first, second (median) and third quartiles (box), and the whiskers extend to the highest observation up to 1.5 times the inter-quartile range (IQR = Q3 − Q1). Observations that exceed the whiskers (often called outliers) are also shown.
with respect to seasonal and or inter-annual change, we compared the post-enrichment population with the synchronous population (May–July 2018) in four villages (Zanga, Bako, Dodougou and Dougougue; Fig. 4) 3–7 km from the focal villages. The median of the post-enrichment May–July 2018 was higher than that of the nearby villages (Fig. 4a, c; \(P < 0.02\), quantile regression). Although the maximum value of the \(^2H\) distribution of the population during the onset of rains was lower than that of the nearby villages (Fig. 4c), the proportion of mosquitoes that exceeded the third quartile of the \(^2H\) distribution of the nearby villages was 33.5%, yielding an excess of 8.5%. Notably, no mosquitoes exceeded the 95% UCL of the third quartile (0%; Fig. 4c).

Using FMM to evaluate the homogeneity of the female populations during the onset of rains, we detected evidence for a mixed population with the larger subpopulation (97.5%) having a mean of 136 ppm \( (P < 0.001)\) and variance of 8 ppm\(^2\), and the smaller subpopulation (2.5%) having a mean of 145 ppm \( (P < 0.001)\) and variance of 0.3 ppm\(^2\), which were significantly separated \( (P < 0.0003)\). Box- and whisker plots show the first, second (median) and third quartiles (box), and the whiskers extend to the highest observation up to 1.5 times the inter-quartile range (IQR = Q3 – Q1). Observations that exceed the whiskers (often called outliers) are also shown.

Alternative sources of \(^2H\)-enriched mosquitoes in Thierola

The shrinking gap between pre- and post-enrichment mosquitoes four to eight months after end of marking and the evidence for seasonal

---

**Fig. 4** Evaluation of the proportion of marked mosquitoes during the onset of rains, seven to eight months after end of enrichment.

**a.** Comparison of the pre-enrichment (September 2017) in Thierola (red), M’Piabougou (blue) and nearby villages (3–7 km) (dark green) with corresponding post-enrichment distribution of March–April 2018 and May–July 2018 (15 May–5 July), as well as the late July 2018 (18–30 July), reflecting the first cohort of mosquitoes growing in newly formed larval sites the following season: ‘No enrichment’) by box-and-whisker plots;

**b.** Comparison of the post-enrichment distribution of May–July 2018 with pre-enrichment (September 2017) by histogram showing the median (p50) and third quartile (p75) as explained in Fig. 3;

**c.** Comparison of the post-enrichment distributions of May–July 2018 in focal villages with those of nearby villages (3–7 km away) employing the quartile method described in the paragraph above.

**d.** The composition of the May–July post-enrichment females \( (n = 276)\) using FMM with two subpopulations (initial mean and variance 134 and 8, and 144 and 5, for each subpopulation, respectively). The results support a mixed population with a mean of 136.3 ppm and variance of 5.7 ppm\(^2\) \( (P < 0.001, 81\%)\) and a smaller subpopulation with a mean of 144.2 ppm and variance of 4.5 ppm\(^2\) \( (P < 0.001, 19\%)\), which are significantly separated \( (P < 0.0033)\).
and/or inter-annual variation in $^2$H (Fig. 3c) highlighted the need to characterize the spatio-temporal variability in natural $^2$H. We used mosquito samples representing natural $^2$H across seasons and years from Thierola, nearby (3–7 km) villages and distant (25–120 km) villages (Extended Data Fig. 1). Using quantile regression (Methods and Supplementary Table 1), we estimated seasonal variation at the different sites across quartiles (Fig. 5a). The results revealed a geographical cline in $^2$H levels showing a site-specific pattern, a ‘convergence’ across quartiles (Fig. 3c). Although seasonal dynamics of $^2$H levels showed a site-specific pattern, a ‘convergence’ across sites was observed in March–April, the peak $^2$H level in Thierola (and Ballabougou; Fig. 5a). To further assess whether Niono could serve as the source of the migrants to Thierola (and Ballabougou), confounding the post-enrichment distributions in March–April and/or May–June, these distributions were compared (Fig. 5b,c). In March–April, these populations exhibited similar distributions, except mild excess of higher $^2$H values in the focal villages (Thierola; Fig. 5b). However, in May–June, pronounced differences precluded the possibility of mass migration from Niono into Thierola, thus providing decisive support for aestivation.

In this eight-month mosquito mark–capture study, using $^2$H stable isotopes, we aimed to evaluate the contribution of aestivation to the persistence of Sahelian Anopheles throughout the dry season by the ultimate evidence: tracking mosquitoes marked at the end of the wet season until the beginning of the following wet season. By the end of the enrichment phase (November 2017), the proportions of An. coluzzii marked in Thierola and M’Plabougou were 56% and 10%, respectively, with even higher percentages of An. gambiae and An. arabiensis. This difference between species is consistent with previous results suggesting that An. coluzzii density plummet indoors and in larval sites during this period, presumably as they depart to some undiscovered shelters and/or prepare for aestivation by sugar feeding\(^{2,3,25}\), while An. gambiae and An. arabiensis attain their peak densities\(^{1,4,15}\). On the basis of the $^2$H degradation model established previously to assess the

---

**Table 1.** Proportion of marked mosquitoes (%)**

| Month       | Focal villages | Niono (adj) |
|-------------|----------------|-------------|
| Mar–Apr     | N = 70, Med = 137.6 | N = 50, Med = 139.5 |
| May–Jun     | N = 96, Med = 137.7 | N = 219, Med = 136.5 |
| Jul–Sep     | N = 219, Med = 136.5 | N = 219, Med = 136.5 |
| Oct–Nov     | N = 219, Med = 136.5 | N = 219, Med = 136.5 |

Proportion of marked mosquitoes (%):

- **Mar–Apr:**
  - Focal villages: 50%
  - Niono (adj): 50%

- **May–Jun:**
  - Focal villages: 50%
  - Niono (adj): 70%

- **Jul–Sep:**
  - Focal villages: 50%
  - Niono (adj): 70%

- **Oct–Nov:**
  - Focal villages: 50%
  - Niono (adj): 70%
expected $^4$H signal seven months after enrichment\textsuperscript{29}, we had planned and achieved an increased initial $^4$H concentration to values almost double those of the pilot study. This increase served to "lift" the decay curves and extend marking detectability to over seven months. Several months post-enrichment, the mosquito $^4$H levels in the focal villages had declined as anticipated, and although some values above pre-enrichment levels persisted until July 2018, conservatively estimating the proportion of $^4$H marked mosquitoes required use of several independent methods. Early after the end of enrichment (December–January), a small sample available from Thierola ($n = 7$) revealed that 29% of the An. coluzzii population in the combined focal villages was marked. At the onset of rains of the next wet season (May–June 2018), seven months after the $^4$H enrichment, 7% of the population had $^4$H values above the pre-enrichment maximum, and the excess over the third quartile of the pre-enrichment population was 21% (September 2017; Fig. 3a,b). Although an "ad hoc test," the test based on the third quartile is especially useful to address the effect of $^4$H degradation in the marked mosquitoes over many months and is robust given a sample size >60. Testing the excess of mosquitoes above the third quartile of the pre-enrichment population relies on the same baseline justification and complements the previous test based on the maximum natural $^4$H value of the reference population.

Concomitant comparison with the neighbouring villages (3–7 km away) revealed 9% and 10% excess over the 75th and the 50th percentiles (third quartile and median), respectively, although only the difference between the medians was significant ($P < 0.02$, quantile regression; Fig. 4a,c). Finally, FMM analysis supported a mixed population with 2.5% representing a subpopulation with elevated mean $^4$H compatible with our predictions (Fig. 4d). We have carried out the FMM tests in populations we suspected consisted of mixed subpopulations due to marking. In the neighbouring villages 3–7 km from our focal villages, we suspected that local movements of mosquitoes from shelters to nearby villages could potentially generate subpopulations of marked and unmarked mosquitoes, albeit to a lesser degree. However, during this period (late June–July), the predominant winds are from the south, where natural $^4$H levels are lower than in Thierola (which is lower than in Niono). Therefore, migrants would affect the left tail distribution of all the villages in the area. However, our FMM results (and quantile regression results) show that the heterogeneity was in the right tail. Migration from the north would require that mosquitoes would fly against predominant winds for over 100 km, which is unlikely. The consistent trend of decline in the estimates of aestivators adds credence to the results and points to dispersal among neighbouring villages as a factor that "diluted" the number of aestivators at the focal villages after the onset of rains (see the following). Altogether, these results establish the presence of mosquitoes with elevated $^4$H levels over that expected naturally (marked mosquitoes) and thus support local persistence of a substantial part (if not all) of An. coluzzii mosquitoes after the long dry season in the Sahel.

Only the results for the late dry-season peak (March–April 2018) provided less-conclusive support for aestivation, despite finding that 24% exceeded the maximum of the pre-enrichment period (10% > 145 ppm, Fig. 2a,b) and 33% represented excess over the third quartile of the pre-enrichment distribution (Fig. 3b), because comparison with March–April 2016 suggested that a seasonal variation in $^4$H levels could also have given rise to a similar distribution. In this comparison, 6.5% of post-enrichment mosquitoes exceeded the maximum of the 2016 distribution, but no excess population was found beyond the 2016 third quartile (Fig. 3c). The results of the FMM provided further support for aestivation by indicating a mixed (female) population with 19% having a mean of 144.2 ppm ($P < 0.001$), which were statistically separated ($P < 0.0033$; Fig. 3d). Therefore, although the March–April 2018 results strongly support aestivation, they cannot rule out long-range migration.

The degradation of $^4$H values in field $^4$H-enriched mosquitoes over four to eight months\textsuperscript{30} and the realization of a spatio-temporal variation in natural $^4$H level led us to use multiple approaches to evaluate the proportion of marked mosquitoes with elevated $^4$H values in the focal villages. Taking the mean value of these estimates (pooling the focal villages) for each period to assess the temporal trend revealed that during the late dry-season peak (March–April), 17% of An. coluzzii were marked, and 6.1% were still marked by the onset of the rains of the subsequent wet season (15 May–5 July; Supplementary Table 1). Given a starting fraction of 33% (see the preceding and Supplementary Table 1), these results entail that 18% of the mosquitoes at the onset of the new rains were aestivators that had survived the seven- to eight-month dry season (Fig. 6). This trend indicates an accelerated decline in marked mosquitoes after February (Fig. 6), which may be accounted for by increased dilution due to inter-village exchange (see the following). Previous evidence based on mosquito hotspots in Thierola during the dry and wet seasons suggested that mosquitoes shelter outside the boundary of the village and probably within one to a few kilometres from the village\textsuperscript{31}. Thus, movement between neighbouring villages would result in underestimating the actual fraction of aestivators because of the influx of unmarked immigrant aestivators from neighbouring villages and the loss of marked emigrant mosquitoes who moved into neighbouring villages. Indeed, occasional movement between neighbouring villages was documented in previous studies in our area\textsuperscript{14} and elsewhere in the Sahel\textsuperscript{15–17}. Using a different marking method\textsuperscript{36}, mosquitoes in the same area dispersing between villages 3–7 km and even 12 km apart have been recaptured in a MRR study, indicating frequent inter-village exchanges (Dao et al. unpublished). The mosquito mean and median $^4$H values in the four surrounding villages (3–7 km from the focal villages; Extended Data Fig. 2) were significantly lower than those of the focal villages (Fig. 3a,c), yet the mosquito with the highest $^4$H value during that period was found in Zanga, 3 km north of Thierola (Fig. 4a), consistent with mosquito exchanges between these villages. The accelerated reduction in marked mosquitoes (Fig. 6) after January coincides with the period of change in the village hotspots that supported local movement of mosquitoes during the late dry season in Thierola\textsuperscript{37}. Therefore, the estimate of 18% represents the minimum proportion of aestivators as it does not consider marked An. coluzzii
that dispersed to nearby villages or those unmarked from nearby villages that dispersed from neighbouring villages into the focal ones.

Considering the hypothesis that the elevated $^2$H values can be explained without marked aestivating mosquitoes by either (1) seasonal change in $^2$H in focal (and other Sahelian) populations or (2) mass migration from distant localities where background $^2$H levels are higher, we have included additional comparisons. In the absence of monsoon rains during the dry season, evaporative transpiration of the common hydrogen occurs at a higher rate than that of the (heavier) deuterium, resulting in seasonal fluctuation of natural $^2$H values in bodies of overground and underground water. Variation in natural $^2$H in Thierola, Ballabougou and Sokourani (Niono) using the median $^2$H followed accordingly (Figs. 4a and 5). The available literature on variation in $^2$H focuses on spatial variation on scales of many tens, hundreds, and thousands of kilometres, but we are not aware of studies showing variation on smaller scales (several to a few tens of kilometres). The stability of available $^2$H maps (isoscases) over consecutive years and seasons allows using natural variation as markers of provenance.

Moreover, we specifically tested that variation in our focal village Thierola by comparing the pre-marked (September) natural levels in 2017 with the postmarked population in July 2018 (four weeks after the first rains) and found nearly identical distributions (Fig. 4a,b). In the absence of continuously measuring $^2$H in villages far enough to exclude migration but close enough to reflect local $^2$H (for example, ~20 km), one cannot accurately estimate seasonal and inter-annual change in $^2$H in our focal village mosquitoes after enrichment (see Extended Data Fig. 4 and the preceding about expectation for minimal change). Since this is not available, and no other information on seasonal variation in insects in this region is available, the next best thing is to contrast the post-marking populations with the pre-marking population, especially because this generation is closest to that of the generation of (putative) aestivators.

Although a plausible hypothesis, the lower median $^2$H in neighbouring villages compared with the focal villages (Fig. 4a,c) does not reconcile with either hypothesis because the neighbouring villages would be expected to exhibit similar $^2$H levels if either hypothesis were true. The migration hypothesis also requires mosquitoes to fly tens or hundreds of kilometres downwind from an area of exceptionally high density. To the best of our knowledge, only the rice irrigation area of Niono (~140 km northeast of Thierola and Sokourani (~6.050° W, 14.217° N) and Dougouguele (7.170 W, 13.611° N) was conducted in the neighbouring villages (3–7 km from Thierola): Zanga (7.220° W, 13.686° N), Bako (7.265° W, 13.643° N), Dodougou (7.160° W, 13.646° N) and Dougouguele (7.170 W, 13.611° N; Extended Data Fig. 2). To evaluate geographical variation in $^2$H background levels of adult mosquitoes in the region, indoor collections were also conducted in two distant villages: Ballabougou (7.386° W, 13.860° N), 22 km north northeast of Thierola and Sokourani (6.050 W, 14.217° N; Extended Data Fig. 2), 140 km east northeast of Thierola. Sokourani is located at the Niono rice irrigation area and is referred to as Niono henceforth. To address spatio-temporal variation in natural $^2$H, we also used samples of mosquitoes collected on other studies in Thierola and several other villages between 2015 and 2019.

**Methods**

**Study area**

The fieldwork, including marking and mosquito collection, was conducted in the Sahelian villages of Thierola (7.215° W, 13.658° N) and M’Piabougou (7.191° W, 13.599° N), Mali, which are 6 km apart and are referred to as the focal villages (Extended Data Fig. 2). Collections of adult mosquitoes, without marking by $^2$H enrichment were also conducted in the neighbouring villages (3–7 km from Thierola): Zanga (7.220° W, 13.686° N), Bako (7.265° W, 13.643° N), Dodougou (7.160° W, 13.646° N) and Dougouguele (7.170 W, 13.611° N; Extended Data Fig. 2).

**Stable isotope enrichment of larval sites**

Enrichment of mosquito larval sites in Thierola and M’Piabougou began on 23 September 2017 and 30 September 2017, respectively. Enrichment ended 20 November 2017 (Thierola) and 3 December 2017 (M’Piabougou), when larval sites dried up. The enrichment protocol followed ref. 28. Briefly, 27 larval sites in the focal villages were used for enrichment; some were sections of a larger body of water (natural larval site) that were separated by embankment from the rest of the larval site (see the following). Medium and large natural larval sites were selected such that they would retain rainwater for the longest period possible, and a minimum of three weeks. Sandbag embankments were erected within the larger larval sites to prevent dilution of enrichment material in the total water volume and ensure the target concentrations could be maintained within the compartments. As larval-site water levels receded with time, the embankment locations were adjusted and new ones were erected, increasing the relative area of enrichment and leading to natural increases in $^2$H due to evaporation. We used $^2$H deuterium oxide (99.6% D$_2$O, Cambridge Isotope Laboratories) (also heavy water, $^4$H$_2$O or D$_2$O; d2H or dH) for larval-site enrichment. Initial enrichment using 99.6% D$_2$O aimed to reach 2.5 ml l$^{-1}$ $^2$H on the volume of the water measured using maximum and minimum diameters of the larval site and multiple stakes (as dipsticks) to measure average depth. In addition, we supplemented 0.25% (0.625 ml l$^{-1}$ $^2$H) on a weekly basis to compensate for underground water infiltration. Every three days, we also added 200 ml of a microbiota culture produced from microorganisms harvested from the larval sites and cultured in 1% deuterated larval-site water, as $^2$H-enriched larval diet. Larval-site water volume was measured daily and after each rain compensation dosage of $^2$H was added per the volume of water that was added by the rain at the same concentration (2.5 ml l$^{-1}$ $^2$H).

**Adult mosquito collection**

Adult mosquitoes were collected indoors in both Thierola and M’Piabougou in pre-selected periods: September (mid-wet season)—pre-enrichment; October–November 2017—enrichment phase (late wet season); December–January (early dry season); March–April 2018—late
dry-season peak (late dry season); May–June 2018—onset of rains of the new rainy season; and July—the first cohort of mosquitoes that developed in the newly formed larval sites after the rain (see timeline, Fig. 1). However, because of the late arrival of the first rains in 2018 (June 24), all mosquitoes collected up to the ten-day period following the rain were considered to be either aestivating or migrating adults because there was no time for a new cohort to be produced1,2. Thus, all mosquitoes collected from 15 May until 5 July were pooled to represent the emergence of putative aestivators (or long-distance migrants). The first cohort of mosquitoes that developed in the newly formed larval sites after the rains were collected 18–25 July to ensure minimal overlap between this cohort and the previous population (see timeline, Fig. 1).

Adult mosquitoes (Anopheles sp.) were collected indoors in the morning, using a mouth aspirator, within ~100 houses per village. Collected mosquitoes were identified to species or genus and separated by sex and gonotrophic state before being killed and stored individually over a thin layer of cotton placed over desiccant (silica gel orange, cat. no. 10087, Sigma-Aldrich) in 0.6 ml microcentrifuge tubes. On a few occasions, we used previously collected samples preserved in 75% ethanol.

Following morphological identification12, species identity of the members of the Anopheles gambiae s.l. complex was resolved using a PCR with two legs as template20. This assay enabled identification of An. coluzzii (formerly A. gambiae M form), A. gambiae s.s. (formerly A. gambiae S form) and A. arabiensis without the need for restriction enzymes digest of the PCR product.

Stable isotope analyses
Quantification of 2H enrichment in mosquitoes was previously described in ref. 20. Briefly, desiccated A. gambiae s.l. carcasses were divided into main body parts (head, thorax, abdomen, legs and wings). Only thoraces were included in our analysis as they constituted the largest chitinous mass in the mosquito. The other body parts were either used for molecular taxonomy (legs) or kept for future analyses. In a handful of cases when thoraces were unavailable, a combination of head, wing and legs was used instead. Dry mosquito samples were prepared for isotope-ratio mass spectrometry as follows: mosquito samples (and respective standards; see the following) were exposed to the spectrometry facility’s laboratory environment for at least 72 hours before analysis to facilitate equilibration of exchangeable hydrogen with local atmospheric water vapour. Water-vapour-equilibrated mosquito thoraces were individually weighed in 8 × 5 mm silver capsules (cat. no. D2008. EA Consumables) (mean: 0.25 mg; range: 0.15–0.40 mg). Crushed capsules were interspersed with appropriate standards and blanks approximately every ten samples. Stable hydrogen isotope analyses of wild mosquitoes were performed at the Smithsonian Museum Conservation Institute Stable Isotope Mass Spectrometry reference laboratory on a Thermo Delta V Advantage continuous-flow isotope-ratio mass spectrometer coupled to a Thermo Fisher Scientific. Samples were thermally decomposed to elemental H2 in a ceramic reactor filled with chromium powder at 1,100 °C. Non-exchangeable 2H values (δ2Hnon-ex) were determined via three-point linear calibration1 with three keratin reference materials dispersed every ten samples: CBS (Caribou Hoof Standard, δ2Hnon-ex = −197.0‰) and USGS42 (Tibetan Human Hair, δ2Hnon-ex = −150.2‰) and BARREL-2 (δ2H = +800‰), were included in every run to monitor slope and isotopic drift.

All 2H data are reported in mass-fraction notation, in units of parts per million (ppm), to accommodate the gravimetric mass-fraction enrichment method used in the study and to ensure consistency of reporting for water, dose and tissue results22,23. Conversion from δ2H‰ (per mill) values (relative to Vienna Standard Mean Ocean Water (VSMOW)) to ppm followed the formula:

\[
2H_{\text{ppm}} = (1000 + \delta^{2H}_{\text{VSMOW}}) / 6.420135
\]

Statistical analysis
Sample 2H analysis. Briefly and as detailed in the following, although we cannot measure the natural 2H in the focal villages after enrichment began, we have used multiple baselines using multiple populations and time points that span the natural range covering that of the focal villages (at different seasons) and have conservatively interpreted the results across all comparisons.

Comparison with pre-enrichment values. To identify marked (3H enriched) specimens, we relied on comparing mosquitoes collected as emerging adults from the enriched larval sites during enrichment with those collected indoors just before enrichment started. During enrichment (October–November), a large gap in 2H values (>20 ppm) between the highest value of the pre-enrichment distribution (142 ppm) and lowest value of the marked mosquitoes from enriched larval sites allowed us to classify enriched and non-enriched mosquitoes readily on the basis of 2H values (Fig. 6). The first estimator of the fraction of marked mosquitoes was, therefore, the fraction of mosquitoes whose values exceeded 142 ppm. This approach aims to determine whether we have individuals in the post-marking population that are above this highest value of the natural local-synchronous population just before marking. Since no mosquito breeding can take place during the dry season (for lack of surface waters), all mosquitoes collected during the dry season (and the ensuing rains) are either local aestivators or migrants.

Assessing third-quartile excess. In the subsequent three months (December–February), the gap between the unmarked and the putatively marked mosquitoes decreased as previously documented22,23,28. Because of the expected attrition in 2H over four to six months since marking3, we hypothesized that certain marked mosquitoes whose 2H values decayed enough may accumulate in the upper tail of the population, forming an excess of mosquitoes in that part of the 2H distribution. Accordingly, the proportion of mosquitoes in the post-enrichment population whose 2H values were above the third quartile (the 75th quantile) of the pre-enrichment distribution would be larger than the expected 25%. Our second estimator of the fraction of marked mosquitoes was, therefore, the excess mosquitoes over the expected 25%. A conservative statistical test for that hypothesis is testing whether the proportion of mosquitoes in the post-enrichment distribution exceeded the 95% UCL of the third quartile of the pre-enrichment distribution by more than 25% of the 2H values. The 95% UCL estimate was derived using the distribution-free method implemented by Proc Univariate23. For example, if the fraction above the third quartile of the pre-enrichment population was 47%, we attributed the excess 22% (over the expected 25%) to marked mosquitoes, some of which have degraded 2H values below the 142 ppm cut-off.

To test the difference between quantiles of pre- and post-enrichment distributions, we used quantile regression implemented by Proc Quantreg29, which extends the general linear model for estimating conditional change in the response variable across its distribution as expressed by quantiles, rather than its mean (although the median is similar to the mean in symmetric
distributions). Quantile regression does not assume parametric distribution (for example, normal) of the random error part of the model; thus, it is considered semi-parametric. The benefit of this analysis is that it addresses the low level of enrichment that could be detected in the higher quantiles even when the mean or the median is less affected. The parameter estimates in linear quantile regression models are interpreted as in typical generalized linear models, as rates of change adjusted for the effects of the other variables in the model for a specified quantile.

**FMMs.** The third method to estimate the fraction of marked mosquitoes in a population, which is independent of a reference subpopulation, was using FMMs. We hypothesized that the post-enrichment population consisted of a subpopulation of natural, non-enriched mosquitoes and of enriched mosquitoes that had blended in with other following \(^{1}H\) degradation in the latter.\(^{1}H\) Finite mixture models estimate the parameters of the component distributions in addition to the mixing probabilities. Finite mixture models are especially useful for estimating multimodal or heavy-tailed densities and for classifying observations on the basis of predicted component probabilities. \(^{1}H\) These models are increasingly utilized in ecological studies (for example, mark–capture and migration) as a specific type of hidden Markov model.\(^{28}\) We assumed that both subpopulations exhibit a normal distribution of \(^{2}H\) values with distinct means and possibly different variances. Analysis was done using PROC FMM, implemented in SAS 9.4, to estimate these parameters of each population and whether there is overall significant support for the blending of these two populations, in which case we used the proportion of the subpopulation with the higher mean as an estimate of marked mosquitoes.

We relied on the three different methods to estimate the fraction of marked mosquitoes, especially after three months or longer post-enrichment, when marking degradation could have been more influential. Because males exhibit slightly higher \(^{2}H\) values than females,\(^{30}\) we excluded males if significant differences were found in the sex composition of the populations being compared. Each method has its strengths and assumptions; thus, the agreement between their results is a measure of the confidence in the overall estimate, which was calculated as the average of all estimators (zeros and negative values included).

We used the inter-quartile range, calculated as the difference between the third quartile (also known as the 75% quantile) and the first quartile (also known as the 25th quantile) as a measure of population spread. The non-parametric skew, defined as (mean – median)/standard deviation, was used as a measure of asymmetry (skewness), which is a third of the Pearson 2 coefficient of skewness because it bounds between -1 and +1 for any distribution.

Except in comparisons with distant sites (Niono and Ballabou-gou), analysis included desiccated specimens preserved in silica gel. However, some of the specimens from distant sites were collected for different projects and preserved in 75% ethanol. Preservation in ethanol was found to increase the mean \(^{2}H\) value of the mosquitoes by 4.67 ppm (Supplementary Table 1) as the ethanol promotes cellular destruction and destabilizes the structure of water, allowing increased differential evaporation of protium\(^{28,43}\). Visual comparisons and correlations of these distributions were carried out before and after adjusting for the preservation method (by subtracting 4.67 ppm) for all specimens preserved in ethanol\(^{28}\) (Fig. 5b,c).

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The dataset used in our analyses can be found at https://doi.org/10.6084/m9.figshare.20387328.v1.

**Code availability**

SAS code associated with these analyses will be made available by T.L. upon request (tlehmann@niaid.nih.gov).

**References**

1. Adamou, A. et al. The contribution of aestivating mosquitoes to the persistence of Anopheles gambiae in the Sahel. Malar. J. 10, 151 (2011).
2. Huestis, D. L. et al. Seasonal variation in metabolic rate, flight activity and body size of Anopheles gambiae in the Sahel. J. Exp. Biol. 215, 2013–2021 (2012).
3. Huestis, D. L. et al. Variation in metabolic rate of Anopheles gambiae and A. arabiensis in a Sahelian village. J. Exp. Biol. 214, 2345–2353 (2011).
4. Lehmann, T. et al. Aestivation of the African malaria mosquito, Anophelbes gambiae in the Sahel. Am. J. Trop. Med. Hyg. 83, 601–606 (2010).
5. Yaro, A. S. et al. Dry season reproductive depression of Anopheles gambiae in the Sahel. J. Insect Physiol. 58, 1050–1059 (2012).
6. Omer, S. M. & Cloudsley-Thompson, J. L. Survival of female Anopheles gambiae Giles through a 9-month dry season in Sudan. Bull. World Health Organ. 42, 319 (1970).
7. Omer, S. M. & Cloudsley-Thompson, J. L. Dry season biology of Anopheles gambiae in the Sudan. Nature 217, 879–880 (1968).
8. Holstein, M. H. Biology of Anophelbes gambiae (1954). World Health Organization.
9. Andrade, C. M. et al. Increased circulation time of Plasmodium falciparum underlies persistent asymptomatic infection in the dry season. Nat. Med. 26, 1929–1940 (2020).
10. Coulibaly, D. et al. Spatio-temporal dynamics of asymptomatic malaria: bridging the gap between annual malaria resurgences in a Sahelian environment. Am. J. Trop. Med. Hyg. 27, 1761–1769 (2017).
11. Gillies, M. & Wilkes, T. A study of the age-composition of populations of Anophelbes gambiae Giles and A. funestus Giles in north-eastern Tanzania. Bull. Entomol. Res. 56, 237–262 (1965).
12. Gillies, M. T. & De Meillon, B. The Anophilinae of Africa south of the Sahara (Ethiopian Zoogeographical Region) (Johannesburg: South African Institute for Medical Research, 1968).
13. Dao, A. et al. Signatures of aestivation and migration in Sahelian malaria mosquito populations. Nature 516, 387–390 (2014).
14. Thomson, J. G. Malaria in Nyasaland. Proc. R. Soc. Med. 28, 391–404 (1934).
15. Huestis, D. L. et al. Windborne long-distance migration of malaria mosquitoes in the Sahel. Nature 574, 404–408 (2019).
16. Lambert, B., North, A., Burt, A. & Godfray, H. C. J. The use of driving endonuclease genes to suppress mosquito vectors of malaria in temporally variable environments. Malar. J. 17, 154 (2018).
17. Verhulst, N. O., Loonen, J. A. C. M. & Takken, W. Advances in methods for colour marking of mosquitoes. Parasit. Vectors 6, 200 (2013).
18. Hagler, J. R. & Jackson, C. G. Methods for marking insects: current techniques and future prospects. Annu. Rev. Entomol. 46, 511–543 (2001).
19. Hamer, G. L. et al. Dispersal of adult culex mosquitoes in an urban West Nile virus hotspot: a mark–capture study incorporating stable isotope enrichment of natural larval habitats. PLoS Negl. Trop. Dis. 8, e2768 (2014).
20. Hamer, G. L. et al. Evaluation of a stable isotope method to mark naturally-breeding larval mosquitoes for adult dispersal studies. J. Med. Entomol. 49, 61–70 (2012).
21. Opiyo, M. A. et al. Using stable isotopes of carbon and nitrogen to mark wild populations of Anopheles and Aedes mosquitoes in south-eastern Tanzania. PLoS ONE 11, e0159067 (2016).

22. Hood-Nowotny, R., Mayr, L. & Knols, B. Use of carbon-13 as a population marker for Anopheles arabiensis in a sterile insect technique (SIT) context. Malar. J. 5, 6 (2006).

23. Hood-Nowotny, R. & Knols, B. G. J. Stable isotope methods in biological and ecological studies of arthropods. Entomol. Exp. Appl. 124, 3–16 (2007).

24. Hood-Nowotny, R. et al. Intrinsic and synthetic stable isotope marking of tsetse flies. J. Insect Sci. 11, 79 (2011).

25. Atzrodt, J., Derdau, V., Kerr, W. J. & Reid, M. Deuterium- and tritium-labelled compounds: applications in the life sciences. Angew. Chem. Int. Ed. 57, 1758–1784 (2018).

26. Copia, L., Wassenaar, L. I., Belachew, D. L. & Araguás-Araguás, L. J. Comparative evaluation of 1H- versus 3H-based enrichment factor determination on the uncertainty and accuracy of low-level tritium analyses of environmental waters. Appl. Radiat. Isot. 176, 109850 (2020).

27. Begon, M., Harper, J. & Townsend, C. Ecology: Individuals, Populations and Communities (Blackwell Science, 1996).

28. Faiman, R. et al. Marking mosquitoes in their natural larval sites using 3H-enriched water: a promising approach for tracking over extended temporal and spatial scales. Methods Ecol. Evol. 10, 1274–1285 (2019).

29. Florin, M. Chemical Zoology: Arthropoda Part B (Academic Press, 2014).

30. Hackman, R. H. & Goldberg, M. Studies on chitin VI. The nature of alpha- and beta-chitins. Aust. J. Biol. Sci. 18, 935–946 (1965).

31. Faiman, R. et al. Quantifying flight aptitude variation in wild Anopheles gambiae in order to identify long-distance migrants. Malar. J. 19, 263 (2020).

32. Huestis, D. L. & Lehmann, T. Ecophysiology of Anopheles gambiae s.l.: persistence in the Sahel. Infect. Genet. Evol. 28, 648–661 (2014).

33. Lehmann, T. et al. Seasonal variation in spatial distributions of Anopheles gambiae in a Sahelian village: evidence for aestivation. J. Med. Entomol. 51, 27–38 (2014).

34. Costantini, C. et al. Density, survival and dispersal of Anopheles gambiae complex mosquitoes in a West African Sudan savanna village. Med. Vet. Entomol. 10, 203–219 (1996).

35. Toure, Y. T. et al. Mark-release-recapture experiments with Anopheles gambiae s.l. in Banambani Village, Mali, to determine population size and structure. Med. Vet. Entomol. 12, 74–83 (1998).

36. Faiman, R. et al. A novel fluorescence and DNA combination for versatile, long-term marking of mosquitoes. Methods Ecol. Evol. https://doi.org/10.1111/2041-210X.13592 (2021).

37. Brattström, O., Bensch, S., Wassenaar, L. I., Hobson, K. A. & Åkesson, S. Understanding the migration ecology of European long-distance migrants. Environ. Biol. Res. 23, 720–729 (2010).

38. Hobson, K. A., Jingui, H., Ichikawa, Y., Kusack, J. W. & Anderson, R. C. Long-distance migration of the globe skimmer dragonfly to Japan revealed using stable hydrogen (δ2H) isotopes. Environ. Entomol. 50, 247–255 (2020).

39. Schilling, E. G. et al. Phenological and isotopic evidence for migration as a life history strategy in Aeshna canadensis (family: Aeshnidae) dragonflies. Entomol. Ecol. 46, 209–219 (2021).

40. Girard, P., Hillaire-Marcel, C. & Oga, M. S. Determining the recharge mode of Sahelian aquifers using water isotopes. J. Hydrol. 197, 189–202 (1997).

41. Güttler-Expósito, C., Ramírez, F., Afán, I., Forero, M. & Hobson, K. A. Toward a deuterium feather isoscape for sub-Saharan Africa: progress, challenges and the path ahead. PLoS ONE https://doi.org/10.1371/journal.pone.0135938 (2015).

42. Lutz, A., Thomas, J. M. & Panorska, A. Environmental controls on stable isotope precipitation values over Mali and Niger, West Africa. Environ. Earth Sci. 62, 1749–1759 (2011).

43. Risi, C. et al. Understanding the Sahelian water budget through the isotopic composition of water vapor and precipitation. J. Geophys. Res. Atmos. 115, 1–23 (2010).

44. Tremoy, G. et al. A 1-year long δ18O record of water vapor in Niamey (Niger) reveals insightful atmospheric processes at different timescales. Geophys. Res. Lett. 39, 1–5 (2012).

45. Terzer-Wassmuth, S., Wassenaar, L. I., Welker, J. M., Araguás-Araguás, L. J. Improved high-resolution global and regionalized isoscapes of δ18O, δ2H and δ-excess in precipitation. Hydrol. Process. 35 (2021).

46. Hobson, K. A. et al. A multi-isotope (δ13C, δ15N, δ2H) feather isoscape to assign Afrotropical migrant birds to origins. Ecosphere 3, art44 (2012).

47. Diuk-Wasser, M. A. et al. Effect of rice cultivation patterns on malaria vector abundance in rice-growing villages in Mali. Am. J. Trop. Med. Hyg. 76, 869–874 (2007).

48. Sogoba, N. et al. Malaria transmission dynamics in Niono, Mali: the effect of the irrigation systems. Acta Trop. 101, 232–240 (2007).

49. Florio, J. et al. Diversity, dynamics, direction, and magnitude of high-altitude migrating insects in the Sahel. Sci. Rep. 10, 20523 (2020).

50. Wilkins, E. E., Howell, P. I. & Benedict, M. Q. IMP PCR primers detect single nucleotide polymorphisms for Anopheles gambiae species identification, Mopii and Savanna rDNA types, and resistance to dieldrin in Anopheles arabiensis. Malar. J. 5, 125 (2006).

51. Wassenaar, L. I. & Hobson, K. A. Comparative equilibration and online technique for determination of non-exchangeable hydrogen of keratins for use in animal migration studies. Isotopes Environ. Health Stud. 39, 211–217 (2003).

52. Chesson, L. A., Podlesak, D. W., Cerling, T. E. & Ehleringer, J. R. Evaluating uncertainty in the calculation of non-exchangeable hydrogen fractions within organic materials. Rapid Commun. Mass Spectrom. 23, 1275–1280 (2009).

53. Schimmelmann, A. Determination of the concentration and stable isotopic composition of nonexchangeable hydrogen in organic matter. Anal. Chem. 63, 2456–2459 (1991).

54. Speakman, J. Doubly Labelled Water: Theory and Practice (Chapman & Hall, 1997).

55. Base SAS 9.4 Procedures Guide (SAS Institute, 2015).

56. Cade, B. S. & N. B. A. Gentle introduction to quantile regression for ecologists. Front. Ecol. Environ. 1, 412–420 (2003).

57. SAS/STAT® 15.1 User’s Guide (SAS Institute, 2018).

58. Mcclintock, B. T. et al. Uncovering ecological state dynamics with an expanded temporal and spatial scales. Proc. Natl Acad. Sci. USA 115, 10985–109850 (2017).

59. Ventura, M. & Jeppesen, E. Effects of fixation on freshwater diatom distribution patterns and on stable isotopes of carbon and nitrogen. Geophys. Res. Lett. 39, 1–5 (2012).

60. Issam, M., Naulet, N., Martin, M. L. & Martin, G. J. A site-specific and multielement approach to the determination of liquid–vapor fractionation parameters: the case of ethanol and octane. J. Phys. Chem. 94, 8303–8309 (1990).

61. Linderstrøm-Lang, C. U. & Vaslov, F. Isotope effect on the vapor pressures of water–ethanol and deuterium oxide–ethanol–d mixtures. J. Phys. Chem. 72, 2645–2650 (1968).

62. Ventura, M. & Jeppesen, E. Effects of fixation on freshwater invertebrate carbon and nitrogen isotopic composition and its arithmetic correction. Hydrobiologia 632, 297–308 (2009).

Acknowledgements

We thank C. Barillas-Mury, J. Ribeiro, K. A. Hobson, L. I. Wassenaar, G. Hamer, and D. X. Soto for ideation and critical reading of earlier versions of this manuscript. Special thanks is extended to C. A. M. France from The Smithsonian Museum Support Center and...
J. Matthews of U.C. Davis for help with IRMS work. We thank Dr. T. Wellems for his continuing support of our work. We thank F. Bathily, L. Juompan, M. Sullivan, A. Laughinghouse, K. Lee and S. Moretz for logistic support. We thank the residents of the villages of Thierola and M’Piabougou for their cooperation and hospitality. A special thanks goes to our spouses and families for their endurance, dedication and unwavering support throughout this endeavour. This study was supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD.

Author contributions
Conception and study design were by R.F., A.S.Y., A.D. and T.L. Fieldwork and sample collection were done by A.D., A.S.Y., M.D., D.S., Z.L.S. and O.Y. Mosquito identification and sample preparation were handled by R.F., L.M.V., L.C.G., A.R.C. and C.K. Data acquisition (IRMS) was by R.F., A.S.Y., A.D. and T.L. Data interpretation and analysis were done by R.F., A.S.Y., A.D., B.J.K. and T.L. Manuscript drafting was by R.F., A.S.Y. and T.L. Manuscript revision was by R.F., L.M.V., L.C.G., A.R.C., C.K., B.J.K., A.D., A.S.Y., M.D., D.S., Z.L.S., O.Y. and T.L.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41559-022-01886-w.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41559-022-01886-w.

Correspondence and requests for materials should be addressed to Roy Faiman.

Peer review information Nature Ecology & Evolution thanks Francesco Baldini and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© This is a U.S. Government work and not under copyright protection in the US; foreign copyright protection may apply 2022.
Extended Data Fig. 1 | Map of study area. Map of study area including its location (black rectangle) on a map of Mali (inset). Location of the focal villages (red) surrounded by nearby villages (green) and distant villages (blue) is shown. Maps plotted using SAS 9.4 which is licensed to include maps from Gfk GeoMarketing GmbH (inset) and Open Street map.
Extended Data Fig. 2 | Thierola and M'Piabougou satellite images. Thierola (top) and M'Piabougou (bottom) satellite images; houses (black empty circles) and larval sites (Navy-blue dots). Image data from Google (©2022), Maxar Technologies and CNES/Airbus.
Extended Data Fig. 3 | Residual marking. Differences between pre-enrichment mosquitoes from Thierola collected indoors (September 2017: PreEnrContInd), experimentally marked mosquitoes collected as pupae and stage-4 instar larvae from enriched larval sites during enrichment (LV1Enrich and LV5Enrich), and naturally occurring pupae and stage-4 instar larvae collected after the first rain the following year (July 2018: LV1PostEnrich and LV5PostEnrich). Sample size is shown in blue, and green reference lines show the range of natural $^3$H levels as in Fig. 2.
Extended Data Fig. 4 | Natural temporal variation within and between population in median δ²H. Natural temporal variation within and between population in median δ²H (Y axis), spread of δ²H as measured by the inter-quartile range (bubble size), and skewness measured by the non-parametric skewness (values inside bubbles; blue denotes negative skewness and yellow and red denote weaker and stronger positive skewness, respectively as seen in scale bar). Sampling years (August to July) are shown on each line and the letters T and M denote samples for Thierola and M’Piabougou.
Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- **n/a**
- **Confirmed**

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

- **Data collection** No software was utilized for data collection.
- **Data analysis** Data was analyzed using SAS 9.4 licensed to the NIH.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All raw data will be uploaded to the journal's preferred data repository upon paper publication.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Study description | We used hydrogen stable isotope enrichment in natural mosquito breeding sites to mark the village population of Anopheles gambiae and track them throughout the seven month-long dry season to quantify the contribution of aestivation to the wet season population. |
|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Research sample   | All major larval sites within two Sahelian villages were enriched with 2H providing an average of 33% mosquitoes within the population of both villages marked with stable isotopes above background levels. IRMS analysis was conducted on a total of 2586 wild mosquitoes collected in the two focal villages, five surrounding villages, dry and wet season and between the years 2015-2019. |
| Sampling strategy | Wild mosquitoes were mouth-aspirated within village houses; all houses of the focal villages, and 40-80 houses of the surrounding villages depending on their respective size. Mosquitoes were registered for sex, gonotrophic stage and collection location and date and were preserved in desiccant or in 80% ethanol for future analyses. During the dry season very few mosquitoes were collected as to not impinge on the aestivating population. |
| Data collection   | Mosquitoes were collected by qualified entomologists from MRTC/ICER Mali as well as local village collectors trained by our Mali team. Mosquitoes were processed for IRMS analysis in the US and stable isotope data was obtained by the Smithsonian Museum Support Center in Washington DC and analyzed by the authors of this paper at NIH and ICER-Mali. |
| Timing and spatial scale | Field mosquitoes were collected between September 2017 and July 2018 to cover both wet, dry and following wet seasons in the focal villages. Previous mosquito collections from all villages from 2015-2019 were included to check for geographical annual and season stable isotope variations. |
| Data exclusions   | IRMS analysis was optimized initially, where it was determined that whole mosquito 2H values were skewed by an access of soft tissue which promoted natural hydrogen exchange. It was determined that mosquito thoraces were the optimal for the analysis and all whole-body samples were removed from the final analysis. |
| Reproducibility  | To confirm stable isotopic results mosquitoes were analyzed initially in two separate IRMS facilities in Washington DC (Smithsonian) and at UC Davis. Both independent facilities agreed on results and the lion part of the work was further conducted at the Smithsonian facility. |
| Randomization    | N/A. All data were used. |
| Blinding         | N/A. Mosquito sub-samples were split and testes independently by both IRMS facilities. See above. |
| Did the study involve field work? | Yes | No |

Field work, collection and transport

Field conditions

Field operations were carried out in the Sahel of Mali. The region is rural, characterized by scattered villages with traditional mud-brick houses, surrounded by fields. A single growing season (June–October) allows the farming of millet, sorghum, maize, and peanuts, as well as subsistence vegetable gardens. Over 90% of the annual rains fall during this season (~550mm). Cattle, sheep, and goats graze in the savanna that consists of grasses, shrubs, and scattered trees. The rains form small puddles and larger seasonal ponds that usually are totally dry by the end of November. From November until May, rainfall is absent or negligible (total precipitation < 50mm), and by December through most of May, water is available only in deep wells. Temperatures peak (night high >30°C) during the late dry season (March-May) and reach their minima (night near 15-20°C) during the early dry season (December-February). Throughout the dry season (December-May) relative humidity is low (25-15%) with few short exceptions, whereas during the wet season (June-November) it is higher (60-90%) based on the monsoon rains. In the rest of the year, the temperatures are intermediate and depend on monsoon rains. No electricity or water pipes are available in the villages. In the rice cultivation area of Niono rice paddies are irrigated year-round by river water and mosquito breeding is constant.

Location

The field-work including marking and mosquito collection was conducted in the Sahelian villages of Thierola (-7.215 E, 13.658 N) and M’Piaibougou (-7.191 E, 13.599 N), Mali, which are 6 km apart and are referred to as the focal villages (Fig. S1). Collections of adult mosquitoes, without marking by 2H spiking were also conducted in the neighboring villages (3-7 km from Thierola): Zanga (-7.220 E, 13.599 N), Bako (-7.265 E, 13.643 N), Dodougou (-7.160 E, 13.646 N) and Douougoue (-7.170 E, 13.611 N, Fig. S1). To evaluate geographical variation in 2H background levels (natural) of adult mosquitoes in the region, indoor collections were also conducted in two distant villages: Ballalougou (-7.386 E, 13.860 N), 22 km NNE of Thierola and Sokourani (-6.050 E, 14.217 N, Fig. S1), 140 km ENE of Thierola. Sokourani is located at the Niono rice irrigation area and is referred to as Niono hence forth.

Access & import/export

All the work was coordinated and carried out by the Malaria Research and Training Center of Mali (MRTC, now the Mali ICEMR).
Specimens were exchanges based on existing agreements for scientific cooperation between MRTC and NIH. Dead and preserved insects were transferred between countries following existing procedures.

We believe that indoor sampling and predator removal from larval sites had minimal effect on diversity and biological systems being sampled given the fraction of the area actually sampled. Deuterium is non-toxic and is found naturally in any environment as well as used in medical practices and not known to cause any measurable harm in moderate doses.

### Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a                              | n/a     |
| Involved in the study            | Involved in the study |
| ☒ Antibodies                     | ☒ ChIP-seq |
| ☒ Eukaryotic cell lines          | ☒ Flow cytometry |
| ☒ Palaeontology and archaeology  | ☒ MRI-based neuroimaging |
| ☒ Animals and other organisms    |         |
| ☒ Human research participants    |         |
| ☒ Clinical data                  |         |
| ☒ Dual use research of concern   |         |

### Animals and other organisms

Policy information about [studies involving animals: ARRIVE guidelines](#) recommended for reporting animal research

| Laboratory animals | The study did not involve laboratory animals |
|--------------------|--------------------------------------------|
| Wild animals       | Only Anopheles mosquitoes are included in this paper. Other insects were also collected and will be summarized separately. |
| Field-collected samples | All mosquitoes collected by smooth aspirators were preserved immediately in either desiccant or 80% ethanol. They were kept in field conditions for several days before they were moved to a laboratory freezer in Bamako, Mali. |
| Ethics oversight   | No ethical approval is required for sampling mosquitoes indoors. Local village committees approved and supervised usage of deuterium-oxide in the mosquito larval sites in each of the villages where marking took place. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.