Evaluating the contribution of aestivation to the persistence of malaria mosquitoes through the Sahelian dry season using stable isotopes

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Abstract

Data suggests the malaria vector *Anopheles coluzzii* persists in the Sahel by dry-season aestivation though its specific contribution compared with alternative strategies, i.e., migration is unknown. We have marked *Anopheles* mosquitoes using deuterium (^2H) to assess the contribution of aestivation to persistence of mosquitoes through the seven-month dry season. If local aestivation is the only way *An. coluzzii* persists, the frequency of marked mosquitoes should remain stable throughout, whereas finding no marked mosquitoes would be evidence against aestivation. Larval sites were enriched with ^2H at the end of the 2017 wet season (September) in two Sahelian villages in Mali. We monitored ^2H -enriched populations until the onset of rains (June 2018). By the end of the enrichment period (November 2017), 33% of *An. coluzzii* mosquitoes were strongly marked. Expectedly, ^2H levels in marked mosquitoes degraded over time. We utilized three methods to estimate the fraction of marked mosquitoes in the population. Seven months following enrichment all three methods supported the presence of marked mosquitoes: 7% of the population had ^2H values above the highest pre-enrichment value. An excess of 21% exceeded the expected 25% in the 3rd quartile of the pre-enrichment population. Finally, a finite mixed population model showed 2.5% represented a subpopulation of marked mosquitoes with elevated ^2H , compatible with our predictions. We provide evidence 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, suggesting either mixture among local populations or that *An. coluzzii* utilizes several persistence strategies. The implications for mosquito control and malaria elimination campaigns are highlighted.

Introduction

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 *An. coluzzii* persists in the West African Sahel as aestivating adults, i.e., in a state of summer dormancy typically characterized by suppressed reproduction and/or growth that facilitate extended survival during harsh conditions^{4,5,10-12}, supporting previous studies with similar assertions¹³⁻¹⁵. 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-long 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 consequentially^{2,3}. Survival of *Anopheles gambiae* s.l., the primary malaria vector during the wet months averages 3-6 weeks typically, suggesting no mosquitoes should survive the 6-8 month-long 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 absent⁴⁻⁶. Days after the first rain, but noticeably *before* a new generation of mosquitoes can develop to adulthood, *An. coluzzii* reappear⁴⁻⁶. The 'dry-season malaria paradox' of dry-

season mosquito persistence has been puzzling malariologists and medical entomologist for almost a century⁶⁻⁹.

To date, direct evidence, such as the capture of aestivating adult mosquitoes has remained limited^{4,14}. Interestingly, for a brief period in late March to early April, *An. coluzzii* densities rebound inexplicably⁵. The source of the mosquitoes during this period has been attributed either to local aestivation, or to 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^{5,6}.

Tracking insects over extended time remains a challenge given their small size and large dilution in the unmarked population^{16,17}. Optimal marking should not affect the behavior, development, longevity or reproduction of mosquitoes, simple to apply, persist throughout the duration of the experiment, and should not be prohibitively priced to apply and detect¹⁸. Stable isotopes (e.g., ¹³C and ¹⁵N most commonly) have previously been used to mark and track several insect species, including mosquitoes on a limited spatio-temporal scale¹⁹⁻²⁴ (see Suppl. Info.). 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. Although not tested on mosquitoes in the laboratory for periods beyond 50 days (*An. gambiae* s.l. maximum survival in insectary conditions), ²H (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 mosquitoes²⁵. Hydrogen loss from chitinous tissues caused by deuterium exchange with protium (¹H), enzymatic digestion and hydrolysis, was shown to occur in regions of α-chitin of the cuticle of insects and crustaceans^{26,27}. Despite degradation of the marking over extended time periods, ²H levels in mosquitoes remain above natural levels for an extended duration after experimental enrichment²⁵, and providing an even higher experimental dose may outlast the aestivation period.

Here, we aimed to empirically estimate the contribution of aestivation to the persistence of mosquitoes through the dry season, assessing the fraction of aestivators which 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 ²H-enrichment by the end of the wet season and evaluating the changes in the fraction of marked mosquitoes through the dry season, the late dry season peak, and especially at the onset of the first rain of the subsequent wet season, before a new generation of mosquitoes could have emerged (see timeline graphic, Fig. S1). 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.

Results

Enrichment during late wet season (September-November): Variation between villages and species

Isotopic enrichment of natural larval sites began in late September when all three anopheline vector species are present and ended when all surface waters dried up in late November (see timeline, Fig. S1). 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 composition in Thierola and M'Piabougou before and during enrichment (September-November) was similar (September: exact test $P > 0.066$, October: exact test $P > 0.46$, November: exact test $P > 0.99$, and overall using Cochran-Mantel-Haenszel Statistics: Nonzero correlation_{df=1} = 0.7023, $P > 0.402$, General association_{df=2} = 3.9075, $P > 0.95$) and therefore villages were pooled (Fig. 1a). The highest ^2H value of the pre-enrichment distribution was 142.2 ppm (n=66, Fig. 1c). Mosquitoes from enriched larval sites were identified based on ^2H values above 145 ppm (Fig. 1c and Fig. S4). In Thierola, highly enriched mosquitoes representing marked individuals ($^2\text{H} > 160$ ppm; Fig. 1c) surpassed 40% in October, before any were observed in M'Piabougou (Fig. 1b). By November, 90% of mosquitoes collected indoors in Thierola were highly enriched mosquitoes, but this fraction was near 15% in M'Piabougou (Fig. 1b). Between-species differences in the proportions of marked mosquitoes were detected in both villages with *An. coluzzii* showing lower marking than *A. gambiae* and *A. arabiensis* (Fig. 1b). The effect of experimental enrichment on ^2H values of mosquitoes collected indoors in each village is evident by the large gap between natural ^2H values and the values of some of the mosquitoes after enrichment began (Fig. 1c and S4). The increase in ^2H enrichment levels over time in mosquitoes (November vs. October) reflects the increasing concentration of ^2H in the larval sites (see Methods).

The exceptionally high proportion of marked adults in Thierola (Fig. 1b) is attributed to the fact that during the late wet season, the village has only two major larval sites; one bordering the village from the west and the other, located 1.2 km NE of the nearest house, as opposed to over 15 larval sites in M'Piabougou (Fig. S3), where enrichment started later and progressed slower than in Thierola. Additionally, most mosquitoes in Thierola were collected from houses near the main larval site, whereas the collections in M'Piabougou were scattered across the village. In Thierola, the proportion of marked mosquitoes was 92% in the houses flanking the larval site, ~10-50 m from the water's edge (n=36), 60% in houses ~50-150 m away (n=30), and 20% in the houses ~150-600 m away (n=5) ($\chi^2_{df,2} = 16.5$, $P < 0.001$), reflecting the accumulation of newly emerged marked mosquitoes in the houses nearest to the larval site and their slower dispersion across the village. Accordingly, the adjusted estimate of the fraction of marked *An. coluzzii* mosquitoes in Thierola in November, based on the number of houses per area was 56.4%. In M'Piabougou, on the other hand, the comparable proportion of marked *An. coluzzii* reached 10%.

Tracking marked mosquitoes during the dry season (December-April)

The first half of the dry season (December-February) is characterized by very few mosquitoes found indoors and outdoors, all of which are *An. coluzzii*⁵. To avoid impinging on the aestivating population only a limited sample of anophelines were collected to assess enrichment levels. Because of the expected attrition in ^2H over 4-6 months since marking²⁵⁻²⁷, we hypothesized that certain marked mosquitoes which their ^2H values degraded enough may “accumulate” in the upper tail of the population’s ^2H distribution, thus inflating the expected percentile. Accordingly, the proportion of mosquitoes in the post-enrichment population whose ^2H values were above the 3rd 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 (see Material and 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 a ^2H 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-April⁶, 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^{4-6, 28}.

During the early dry season (December to February), 1-3 months after marking, the ^2H values of mosquitoes were lower than those during the enrichment period (October-November), but two of the seven mosquitoes exceeded the highest ^2H values measured in the pre-enrichment levels (142 ppm, Fig. 2a), indicating they were marked mosquitoes. Therefore, approximately, one third of the mosquitoes in December-February from Thierola (Fig. 2a, ^2H >152 ppm, one from mid-December and another from the end of January) were considered aestivators, or 29% from the focal villages (2 of 7 mosquitoes).

During March-April, 24% of the mosquitoes collected in Thierola exceeded the highest ^2H value of the pre-enrichment period (142 ppm, Figs. 2a and 2b) and ~10% surpassed 145 ppm. As predicted, the proportion of mosquitoes above the 3rd 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 3rd quartile (of the pre-enrichment distribution) was 43%, indicating a significant excess of at least 18% (Fig. 2b).

The shrinking gap between the pre- and post-enrichment values raised the possibility that seasonal variation in natural ^2H 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. 2c). Because only females were available from the 2016 collections, we excluded males from the 2018 sample. Notably, the ^2H distribution of March-April 2016 had higher median and maximum values (137.2 ppm, 146 ppm, Figs. 2b, 2c) than the corresponding values of the pre-enrichment distribution of September 2017 (134.3 ppm 142.2 ppm) and even the post enrichment distribution of March-April 2018 (136.9 ppm, Fig. 2c), suggesting a seasonal and/or inter-annual variation in natural ^2H . Likewise, a lower proportion of post-enrichment mosquitoes exceeded the maximum of the 2016 distribution (6.5%, Fig. 2c) and no

evidence was found for an excess of post-enrichment mosquitoes above the 2016's 3rd quartile (-2%, Fig. 2c).

We used the finite mixed distribution model (FMM; see 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%) with a mean of 136.3 ppm ($P < 0.001$) and variance of 5.7 ppm², and a smaller subpopulation (19%) with a mean of 144.2 ppm ($P < 0.001$) and variance of 4.5 ppm², which were statistically separate ($P < 0.0033$, Fig. 2d).

Tracking marked mosquitoes during the onset of rain (May-June 2018)

The first rain falls typically between mid-May to mid-June, and the first larvae are spotted in the newly formed larval sites 10-20 days after the first rain ^{4,6}. The first rain of 2018 fell on June 24th (see timeline, Fig. S1). Hence, mosquitoes collected from May 15 to July 5th represent adults that in high probability were not mixed with the young adults which developed in the newly formed larval sites. Although 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 July 17th to July 30th represented mostly the new cohort of adults which 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 results ^{4-6,28}. The first *A. arabiensis* and *A. gambiae* were detected in Thierola on June 22nd and July 5th, 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 (May 15th -July 5th) were higher than the pre-enrichment period (September 2017) and their right tail exceeded the highest ²H values measured in the pre-enrichment population (Fig. 3a). As expected, based on aestivation, three weeks later, the ²H median and 3rd quartile dropped in both villages (median difference of 1.6 ppm, $P < 0.0023$ $t_1 = 3.1$; 3rd quartile difference of 2.2 ppm $P < 0.0001$, $t_1 = 4.0$; 0.9 quantile difference of 3.4 ppm $P < 0.022$, $t_1 = 2.3$, quantile regression), representing the first generation of mosquitoes which developed in the newly formed larval sites (late July). Notably, the difference increased at higher quantiles ($P < 0.052$, $\chi^2_2 = 5.94$) indicating that the greatest difference was related to highest ²H values. As expected, 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, $P > 0.53$ $t_1 = 0.7$; 3rd 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, $P > 0.31$ $t_1 = 1.0$; 3rd 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, Figs. 3a and 3b; four mosquitoes exceeded the 145 ppm mark).

Assessment of excess mosquitoes whose ^2H values were above the 3rd quartile of the pre-enrichment population (see Methods and above) 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 3rd quartile of the pre-enrichment distribution, i.e., a statistically significant excess (see Methods). To further evaluate the presence of enriched mosquitoes in the focal villages 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 Dougouguele, Figs. S3, 3a, 3c) 3-7 km from the focal villages. The median of the post enrichment May-July 2018 was higher than that of the nearby villages (Figs. 3a, 3c, $P < 0.02$ 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. 3c), 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 3rd quartile (0%, Fig. 3c).

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%) with a mean of 136 ppm ($P < 0.001$) and variance of 8 ppm², and a smaller subpopulation (2.5%) with a mean of 145 ppm ($P < 0.001$) and variance of 0.3 ppm², which were significantly separated ($P < 0.0001$, Fig. 3d).

Alternative sources of mosquitoes with elevated ^2H values in Thierola

The shrinking gap between pre- and post-enrichment mosquitoes 4-8 months after end of marking and the evidence for seasonal and/or inter-annual variation in ^2H (Fig. 2c) highlighted the need to characterize the spatio-temporal variability in natural ^2H . We used mosquito samples representing natural ^2H across seasons and years from Thierola, nearby (3-7 km) villages, and distant villages (25-120 km) away (Fig. S2). Using quantile regression (Methods, Supp. Table S1), we estimated seasonal variation at the different sites across quartiles (Fig. 4a). The results revealed a geographical cline in mosquitoes with increasing ^2H values towards the north and a seasonal fluctuation in ^2H levels in each location, which was more pronounced in the typical Sahelian villages (4 ppm range) compared with the rice irrigation area of Niono (2.5 ppm range, Fig. 4a). Although seasonal dynamics of ^2H levels showed a site-specific pattern, a “convergence” across sites was observed in March-April – the peak ^2H level in Thierola (and Ballabougou, Fig. 4a). To further assess if 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 (Figs. 4b and 4c). In March-April these populations exhibited similar distributions, except mild excess of higher ^2H values in the focal villages i.e., Thierola (Fig. 4c). However, in May-June, pronounced differences precluded the possibility of mass migration from Niono into Thierola, thus, providing decisive/pivotal support for aestivation.

Discussion

In this novel eight-month long mosquito mark-capture study, using ^2H 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 proportion of *An. coluzzii* marked in Thierola and M'Piabougou was 56% and 10% respectively, and even higher percentages of *An. gambiae* and *An. arabiensis*. This difference between species is consistent with previous results suggesting that *An. coluzzii* density plummets indoors and in larval sites during this period, presumably as they depart to some undiscovered shelters and/or prepare for aestivation by sugar feeding^{6,29,30}, while *An. gambiae* and *An. arabiensis* attain their peak densities⁴⁻⁶. Several months post enrichment, the mosquito ^2H levels in the focal villages had declined as anticipated²⁵ and although some values above pre-enrichment levels persisted until July 2018, conservatively estimating the proportion of ^2H 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 were marked. At the onset of rains of the next wet season (May-June 2018), seven months after the ^2H -enrichment, 7% of the population had ^2H values above the pre-enrichment maximum and the excess over the 3rd quartile of the pre-enrichment population was 21% (September 2017, Figs. 3a and 3b). Concomitant comparison with the neighboring villages (3-7 km away) revealed 9% and 10% excess over the 75th and the 50th percentiles (i.e., 3rd quartile and median) respectively, although only the difference between the medians was significant ($P < 0.02$ quantile regression, Figs. 4a and 4c). Finally, FMM analysis supported a mixed population with 2.5% representing a subpopulation with elevated mean ^2H compatible with our predictions (Fig. 3d). The consistent trend of decline in the estimates of aestivators add credence to the results and points to dispersal among neighboring villages as a factor which “diluted” the number of aestivators at the focal villages after the onset of rains (see below). Altogether, these results establish the presence of mosquitoes with elevated ^2H levels over that expected naturally, i.e., marked mosquitoes, and thus support local persistence of a significant part (if not all) *An. coluzzii* over 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, Figs. 2a and 2b) and 33% represented excess over the 3rd quartile of the pre-enrichment distribution (Fig. 2b), because comparison with March-April 2016 suggested that a seasonal variation in ^2H 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 3rd quartile (Fig. 2c). The results of the FMM provided further support for aestivation by indicating a mixed (female) population with 19% with a mean of 144.2 ppm ($P < 0.001$) which were statistically separated $P < 0.0033$ (Fig. 2d). Therefore, although the March-April 2018 results strongly support aestivation, they cannot rule out long-range migration.

The degradation of ^2H values in field- ^2H -enriched mosquitoes over 4-8 months²⁵ and the realization of a spatio-temporal variation in natural ^2H level led us to use multiple approaches to evaluate the proportion

of marked mosquitoes with elevated ^2H values in the focal villages. Taking the mean value of these estimates (and 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 (May 15 – July 5, table S1). Given a starting fraction of 33% (see above and Table S1), these results entail that 18% of the mosquitoes at the onset of the new rains were aestivators which had survived the 7-8 months long dry season (Fig. 5). This trend indicates an accelerated decline in marked mosquitoes after February (Fig. 5), which may be accounted for by increased dilution due to inter-village exchange (below). 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 kilometers from the village³⁰. Thus, movement between neighboring villages would result in underestimating the actual fraction of aestivators because of the influx of unmarked-immigrant aestivators from neighboring villages and the loss of emigrant-marked mosquitoes who moved into neighboring villages. Indeed, occasional movement between neighboring villages was documented in previous studies in our area^{4,10} and elsewhere in the Sahel^{31,32}. Mosquitoes dispersing between villages 3-7 km, and even 12 km apart have been recaptured in a MRR study using a different marking technique³³ in the same area, indicating frequent inter-village exchanges (Dao et al. unpublished). The mosquito mean and median ^2H values in the four surrounding villages (3-7 km from the focal villages, Fig. S2) were significantly lower than those of the focal villages (Figs. 3a and 3c), yet the mosquito with the highest ^2H value during that period was found in Zanga, 3 km north of Thierola (Fig. 3a), consistent with mosquito exchange between these villages. The accelerated reduction in marked mosquitoes (Fig. 5) after January coincides with the period of change in the village hot-spots that supported local movement of mosquitoes during the late dry-season in Thierola³⁰. Likewise, FMM analysis on the females in nearby villages (Zanga, Bako, Dougouguele and Dodougou) supported two populations, with the smaller population consisting of 1% (not shown), as expected if some migrants from the focal villages dispersed to the surrounding villages. Therefore, the estimate of 18% represents the minimum proportion of aestivators, as it does not consider marked *An. coluzzii* that dispersed to nearby villages, as well as those unmarked from nearby villages which dispersed from neighboring villages into the focal ones.

Considering the hypothesis that the elevated ^2H values can be explained without marked-aestivating mosquitoes either by: (1) seasonal change in ^2H in focal (and other Sahelian) populations, and (2) mass-migration from distant localities where background ^2H levels are higher^{34–36} 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 ^2H values^{37–41}. Variation in natural ^2H in Thierola, Ballabougou and Sokourani (Niono) using the median ^2H followed accordingly (Fig. S5). Although a plausible hypothesis, the lower median ^2H in neighboring villages compared with the focal villages (Fig. 3a and 3c) does not reconcile with either hypothesis because the neighboring villages would be expected to exhibit similar ^2H levels if either hypothesis was true. The migration hypothesis also requires mosquitoes to fly tens or hundreds of

kilometers downwind from an area of exceptionally high density^{5,6}. To the best of our knowledge, only the rice irrigation region of Niono (~140 km NE, or the inner Niger delta near Mopti, ~300 km NE) meets these requisites during March-April^{42,43}, but not during May-June, when the dominant wind direction blows from the South⁴⁴. Importantly, mosquito natural ²H distribution in Thierola was similar to that of Niono only during the late dry season peak (March-April, Fig. 4a), precluding discrimination between aestivation and long-range migration during that restricted period alone.

These mark-capture results including the persistent decline in the fraction of aestivators detected throughout the dry season (Fig. 5) provide compelling evidence that aestivation contributes at least 20% to the persistence of *An. coluzzii* until the onset of rains. Adamou et al.⁵ reached a similar conclusion based on their experiment using selective removal of dry-season mosquitoes. These authors have interpreted their results as evidence for aestivation as the primary if not the only source of the dry season peak. Our results shed light on an evolutionary strategy little is known about in this group of insects and may prove crucial for our understanding of basic mosquito ecology and the future of malaria elimination campaigns.

Materials And Methods

Study area

The fieldwork including marking and mosquito collection was conducted in the Sahelian villages of Thierola (-7.215 E, 13.658 N) and M'Piabougou (-7.191 E, 13.599 N), Mali, which are 6 km apart and are referred to as the focal villages (Fig. S2). Collections of adult mosquitoes, without marking by ²H enrichment were also conducted in the neighboring villages (3-7 km from Thierola): Zanga (-7.220 E, 13.686 N), Bako (-7.265 E, 13.643 N), Dodougou (-7.160 E, 13.646 N) and Dougouguele (-7.170 E, 13.611 N, Fig. S2). To evaluate geographical variation in ²H background levels (natural) of adult mosquitoes in the region, indoor collections were also conducted in two distant villages: Ballabougou (-7.386 E, 13.860 N), 22 km NNE of Thierola and Sokourani (-6.050 E, 14.217 N, Fig. S2), 140 km ENE of Thierola. Sokourani is located at the Niono rice irrigation area and is referred to as Niono hence forth. To address spatio-temporal variation in natural ²H we also used samples of mosquitoes collected on other studies in Thierola and several other villages between 2015-2019.

Stable isotope enrichment of larval sites

Enrichment of mosquito larval sites in Thierola and M'Piabougou began on September 23rd, 2017 and September 30th, 2017 respectively. Enrichment ended November 20th, 2017 (Thierola) and December 3rd, 2017 (M'Piabougou) when larval sites dried up. The enrichment protocol followed²⁵. 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 (below). 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 ^2H due to evaporation. We used ^2H -deuterium-oxide (99.6% D_2O , Cambridge Isotope Laboratories, Inc. Tewksbury, MA) (also: “Heavy water”, $^2\text{H}_2\text{O}$ or D_2O ; d_2H or ^2H in short) for larval site enrichment. Initial enrichment using 99.6% D_2O aimed to reach 2.5ml $^2\text{H}/\text{L}$, based on the volume of the water measured using maximum and minimum diameters of the larval site and multiple stakes (as dipsticks) to measure average depth²⁵. Additionally, we supplemented 0.25% (0.625 ml $^2\text{H}/\text{L}$) 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 ^2H -enriched larval diet²⁵. Larval site water volume was measured daily and after each rain compensation dosage of ^2H was added per the volume of water that was added by the rain at the same concentration (2.5ml $^2\text{H}/\text{L}$).

Adult mosquito collection, preservation, and identification

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. S1). However, because of the late arrival of the first rains in 2018 (June 24), all mosquitoes collected up to the 10-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 produced^{4–6}. Thus, all mosquitoes collected from May 15 until July 5th were pooled to represent the emergence of putative aestivators (or long-distance migrants). The first cohort of mosquitoes which developed in the newly formed larval sites after the rains were collected July 18-25 to ensure minimal overlap between this cohort and the previous population (see timeline, Fig. S1).

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, 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, St. Louis, MO) in 0.6 ml microcentrifuge tubes. On a few occasions we used previously collected samples preserved in 75% ethanol.

Following morphological identification⁴⁵, species identity of the members of the *Anopheles gambiae* s.l. complex was resolved using a PCR with two legs as template⁴⁶. 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 ²⁵. Briefly, desiccated *A. gambiae* s.l. carcasses were divided to 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 IRMS as follows: mosquito samples (and respective standards; see below) were exposed to the IRMS facility's laboratory environment for at least 72 hrs prior to analysis to facilitate equilibration of exchangeable hydrogen with local atmospheric water vapor. Water-vapor equilibrated mosquito thoraces were individually weighed in 8x5 mm silver capsules (Cat. No. D2008. EA Consumables, Inc. Pennsauken, NJ, USA) (mean: 0.25 mg, range: 0.15-0.4 mg). Crushed capsules were interspersed with appropriate standards and blanks approximately every ten samples. Stable hydrogen isotope analyses of wild mosquitos were performed at the Smithsonian Museum Conservation Institute Stable Isotope Mass Spectrometry reference laboratory in Suitland, Maryland, USA on a Thermo Delta V Advantage continuous-flow isotope-ratio mass spectrometer coupled to a Thermo Temperature Conversion Elemental Analyzer via a ConFlo IV interface (Thermo Fisher Scientific, Waltham, MA). Samples were thermally decomposed to elemental H_2 in a ceramic reactor filled with chromium powder at 1100°C. Non-exchangeable $\delta^2\text{H}$ values (i.e. $\delta^2\text{H}_{\text{non-ex}}$) were determined via 3-point linear calibration ⁴⁷ with three keratin reference materials dispersed every 10 samples: CBS (Caribou Hoof Standard, $\delta^2\text{H}_{\text{non-ex}} = -197.0\text{‰}$), USGS42 (Tibetan Human Hair, $\delta^2\text{H}_{\text{non-ex}} = -78.5\text{‰}$), and KHS (Kudu Horn Standard, $\delta^2\text{H}_{\text{non-ex}} = -54.1\text{‰}$). Blanks (empty capsules, crushed) were inserted between each sample to minimize ^2H carryover and purge the reactor between samples. It should be noted that no chitin reference materials with known $\delta^2\text{H}_{\text{non-ex}}$ values were available commercially at the time of analysis. Keratin reference materials were used because the fraction of exchangeable hydrogen atoms in keratin (i.e. $\sim 0.15\text{-}0.20$) is similar to that of chitin ^{48,49}. For natural abundance samples with values encompassed by the reference materials, analytical errors were $\pm 2.0\text{‰}$. Highly enriched samples with values outside the range of linear calibration encompassed by the reference materials may have higher errors estimated at $\pm 5\text{-}10\text{‰}$. Two water reference materials, USGS47 ($\delta^2\text{H} = -150.2\text{‰}$) and BARREL-2 ($\delta^2\text{H} = +800\text{‰}$), were included in every run to monitor slope and isotopic drift.

All ^2H data is reported in mass-fraction notation, in units of parts per-million (ppm), to accommodate for the gravimetric mass-fraction enrichment method used in the study. Conversion from $\delta^2\text{H}\text{‰}$ (per-mil) values (relative to Vienna Standard Mean Ocean Water (VSMOW)) to ppm followed the formula:

$$^2\text{H}_{\text{ppm}} = (1000 + \delta^2\text{H}\text{‰}_{\text{VSMOW}}) / 6.420135$$

Statistical analysis

To identify marked (^2H 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 based on ^2H values (Fig. 1). The first estimator of the fraction of marked mosquitoes was, therefore, the fraction of mosquitoes whose values exceeded 142 ppm. In the subsequent three months (December-February) this gap between the unmarked and the putatively marked mosquitoes decreased as previously documented ²⁵ and ^{26,27}. Because of the expected attrition in ^2H over 4-6 months since marking ²⁵ we hypothesized that certain marked mosquitoes which their ^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 3rd quartile (i.e., 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 and above the expected 25%. A conservative statistical test for that hypothesis is testing if the proportion of mosquitoes in the post-enrichment distribution also exceeded the 95% upper confidence limit (UCL) of the 3rd 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 Univariate ⁵⁰. For example, if the fraction above the 3rd 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 cutoff.

To test the difference between quantiles of pre- and post-enrichment distributions, we used quantile regression implemented by Proc Quantreg ⁵⁰, 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 (though the median is similar to the mean in symmetric distributions). Quantile regression does not assume parametric distribution (e.g., normal) of the random error part of the model, thus it is considered semi-parametric. The benefit of this analysis is that it addresses low level of enrichment that could be detected in the higher quantiles even when the mean or the median are less affected. The parameter estimates in linear quantile regression models are interpreted as in typical GLM, as rates of change adjusted for the effects of the other variables in the model for a specified quantile ⁵¹.

The third method to estimate the fraction of marked mosquitoes in a population, which is independent of a reference subpopulation, was using finite mixed distribution models (FMM). We hypothesized that the post-enrichment population consisted of a subpopulation of natural, non-enriched mosquitoes, and of enriched mosquitoes that had blended into each other following ^2H degradation in the latter ^{26,27}. Finite mixture models estimate the parameters of the component distributions in addition to the mixing probabilities. Finite mixture models are especially useful for estimating multi-modal or heavy-tailed densities and for classifying observations based on predicted component probabilities ⁵². These models are increasingly utilized in ecological studies (e.g. mark-capture and migration) as a specific type of hidden Markov models ⁵³. We assumed that both subpopulations exhibit a normal distribution of ^2H 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. 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 (IQR), calculated as the difference between the 3rd quartile (also known as the 75% quantile) and the 1st quartile (also known as the 25th quantile) as a measure of population spread. The nonparametric skew, defined as $(\text{mean} - \text{median}) / \text{standard deviation}$ 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 (i.e., Niono and Ballabougou), 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 ^2H value of the mosquitoes by 4.67 ppm (Table S1). Visual comparisons of these distributions were carried out after adjusting for the preservation method (by subtracting 4.67 ppm) for all specimens preserved in ethanol (Fig. 4b and 4c).

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Figures

Figure 1

a. Species composition of indoor collections in Thierola and M'Piabougou (pooled) before (July and September) and during the enrichment (October-November). Sample size (blue) and proportion of each species (black) are shown inside bars (percent). Samples were taken from both villages with minimum sample size/village of $n=23$, except in July (Thierola: $n=20$). Differences in composition between villages were not significant: September (exact test $P>0.071$), October (exact test $P>0.46$), November (exact test $P>0.99$) and overall using Cochran-Mantel-Haenszel Statistics (Nonzero correlation/Row mean score difference_{df=1} = 0.7023 $P>0.402$, General association_{df=2} = 3.9075, $P>0.95$). **b.** The change in the proportion of marked mosquitoes ($^2\text{H}>160$ ppm) after enrichment (October-November) in each village by species (colors). Exact tests of heterogeneity among species were run using Monte Carlo estimation (10,000 pseudo samples). Sample size is given in blue. **c.** Distribution of ^2H values in each village before (September) and during enrichment (October-November) by village. Sample sizes are given in blue and reference lines (green, dot-dashed) delimit natural variation and the gap between them and experimentally enriched (marked) mosquitoes (145-160 ppm).

Figure 2

Evaluation of the proportion of marked mosquitoes during the dry season by different methods. **a.** Comparing the post-enrichment distribution of December-February and March-April 2018 with pre-enrichment (September 2017) in Thierola and M'Piabougou by box-whisker plots; **b.** Comparing the post-enrichment distribution of March-April 2018 with pre-enrichment (September 2017) in Thierola by histogram showing the median (Med) and 3rd quartile (3QRT) as well as the 95% upper confidence limit (UCL75) of the 3rd quartile (green dashed vertical lines) of the pre-enrichment distribution. The expected

proportion of the distribution beyond the maximum, 3rd quartile and upper 95% upper confidence limit of the 3rd quartile are shown above the horizontal braces (see text); **c.** Comparing the post-enrichment distribution of March-April 2018 with March-April 2016 in Thierola (females only) using box whiskers (bottom) and histogram (top) and employing the quartile method; **d.** The composition of the post-enrichment females (n=62) using finite mixed distribution model assuming two subpopulations (initial values of mean and variance 134 ppm and 8 ppm², and 144 ppm and 5 ppm², for each subpopulation, respectively (see Methods). The results support a mixed population with of a mean of 136.3 ppm and variance of 5.7 ppm² (P<0.001, 81%), and a smaller subpopulation of a mean of 144.2 ppm and variance of 4.5 ppm² (P<0.001, (19%), which are significantly separated (P<0.0033).

Figure 3

Evaluation of the proportion of marked mosquitoes during the onset of rains, 7-8 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) (green) with corresponding post-enrichment distribution of March-April 2018 and May-Jul 2018 (May 15-July 5), as well as the late July 2018 (July 18-30, reflecting first cohort of mosquitoes growing in newly formed larval sites the following season – 'No enrichment') by box-whisker plots; **b.** Comparing the post-enrichment distribution of May-July 2018 with pre-enrichment (September 2017) by histogram showing the median (p50) and 3rd quartile (p75) as explained in Fig. 2; **c.** Comparing the post-enrichment distributions of May-July 2018 in focal villages and nearby villages (3-7 km away) and employing the quartile method described above; **d.** The composition of the May-July post-enrichment females (n=276) using finite mixed distribution model (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 ppm² (P<0.001, 81%), and a smaller subpopulation of a mean of 144.2 ppm and variance of 4.5 ppm² (P<0.001, 19%), which are significantly separated (P<0.0033).

Figure 4

Spatial and seasonal natural variation in ^2H and evaluating the possibility that mass migration from Niono accounts for the elevated ^2H values in post-enrichment Thierola. **a**. Seasonal natural variation in ^2H in Thierola, Ballabougou, and Niono evaluate over the quartiles of the distributions using quantile regression (Table S1). Bands represent 90% confidence intervals. Note: during October-November, no sample was available from Niono. Evaluating the similarity between the post-enrichment populations from Thierola and the concomitant population from Niono using histograms showing the median (p50) and 3rd quartile (p75, as explained in Fig. 2) in March-April (**b**) and in May-June (**c**). ^2H values of samples from Niono preserved in ethanol were adjusted ([adj]) to correct for preservation artifact.

Figure 5

Change in the proportion of marked *An. coluzzii* over the course of the experiment. Values represent the mean fraction over all estimates (Table S1). Right axis expresses proportions of marked mosquitoes from the total sample and the left axis expresses the corresponding values from the end of the marking period for (November 2017, i.e., 0.33 is the 100%). Fraction values of marked mosquitoes from the end of enrichment are given near each point.

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