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The relationship between insecticide resistance, mosquito age and malaria prevalence in *Anopheles gambiae* s.l. from Guinea

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Insecticide resistance across sub-Saharan Africa may impact the continued effectiveness of malaria vector control. We investigated the association between carbamate and pyrethroid resistance with *Anopheles gambiae* s.l. parity, *Plasmodium falciparum* infection, and molecular insecticide resistance mechanisms in Guinea. Pyrethroid resistance was intense, with field populations surviving ten times the insecticidal concentration required to kill susceptible individuals. The L1014F *kdr*-N1575Y haplotype and I1527T mutation were significantly associated with mosquito survival following permethrin exposure (Prevalence Ratio; PR = 1.92, CI = 1.09–3.37 and PR = 2.80, CI = 1.03–7.64, respectively). Partial restoration of pyrethroid susceptibility following synergist pre-exposure suggests a role for mixed-function oxidases. Carbamate resistance was lower and significantly associated with the G119S *Ace-1* mutation. Oocyst rates were 6.8% and 4.2% among resistant and susceptible mosquitoes, respectively; survivors of bendiocarb exposure were significantly more likely to be infected. Pyrethroid resistant mosquitoes had significantly lower parity rates than their susceptible counterparts (PR = 1.15, CI = 1.10–1.21). Our findings emphasize the need for additional studies directly assessing the influence of insecticide resistance on mosquito fitness.

Malaria remains a leading cause of morbidity and mortality in the tropics, where it is estimated to have resulted in ~445,000 deaths in 2016 alone[1]. Despite considerable reductions in disease burden achieved by scaling-up the provision of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS)[2], long-term effectiveness of both strategies may be jeopardized by widespread emergence of insecticide resistance in mosquito populations[3,4]. In response, commercial manufacturers are developing alternate vector control interventions, as well as optimizing use of existing insecticides through combinations and improved formulations. National Malaria Control Programmes (NMCPs) and international policy makers are also taking action to expand resistance monitoring and surveillance[5]. However, the severity of this threat is currently unknown because there is limited evidence linking the operational failure of available control measures to the presence of resistant mosquito species[6–14].

The efficacy of IRS and LLINs is predicated on their ability to reduce the daily survival rate of *Anopheles* mosquitoes and prevent the completion of parasite development to the infectious stage. One explanation for the sustained effectiveness of LLINs, even in the presence of increased vector tolerance to their insecticidal properties, is that intact nets provide a physical barrier to mosquito feeding. A meta-analysis of field data indicated that treated nets reduced blood feeding and increased mosquito mortality compared to untreated nets, even in areas with the highest levels of resistance[4]. Furthermore, a recent large-scale, multi-country trial reported no association between malaria disease burden and pyrethroid resistance, with evidence that LLINs continued to

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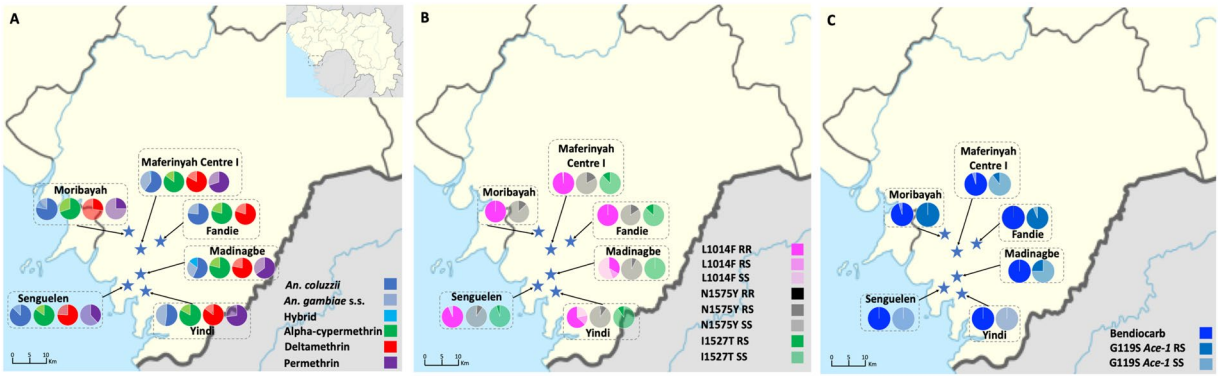

Figure 1. (**A**) Map of Forecariah Prefecture, Guinea displaying proportions of *An. gambiae* s.l. molecular forms and susceptibility levels to the diagnostic dose (1X) of alpha-cypermethrin, deltamethrin and permethrin, measured using CDC bottle bioassays, at six study sites indicated by stars (Fandie, Madinagbe, Maferinyah Centre I, Moribayah, Senguelen and Yindi). Inset map shows the location of Forecariah Prefecture in Guinea. (**B**) Map of Forecariah Prefecture, Guinea displaying frequencies of L1014F, N1575Y and I1527T resistant (R) and wild type (S) alleles. (**C**) Map of Forecariah Prefecture, Guinea displaying susceptibility levels to the diagnostic dose (1X) of bendiocarb, measured using CDC bottle bioassays, and frequency of G119S *Ace-1* resistant (R) and wild type (S) alleles. For all maps, legend colours referring to insecticides have darker shading denoting average mosquito mortality.

provide personal protection across areas of different resistance intensities[15]. Laboratory studies now suggest that fitness costs associated with insecticide resistance may influence malaria transmission either by directly reducing mosquito life span[16] and/or fecundity[17], altering host seeking, feeding and mating behaviours[18–20] or by impairing parasite development inside vectors[21–23]. However, to date, few field studies have directly investigated the impact of insecticide resistance intensity on malaria transmission dynamics.

In Guinea, malaria is a serious public health problem, where the entire population of ~11.7 million people is at risk and the nationwide prevalence is estimated at 15%[24]. Between 2013–2017, the NMCP, with support from the President's Malaria Initiative (PMI) and the Global Fund, have procured and distributed over 27.6 million pyrethroid LLINs[25]. Because the current vector control strategy relies almost exclusively on LLIN use[26], nationwide pyrethroid resistance is of concern[27]. To better inform future malaria control efforts in Guinea, there is a need to characterize levels of operationally-significant insecticide resistance, as well as determine the effect this phenomenon has on the vectorial capacity of local mosquitoes to transmit malaria.

Results

Mosquito abundance and species identification. A total of 3962 female *An. gambiae* s.l. mosquitoes were captured from six sites in Maferinyah subprefecture, in Forecariah Prefecture (Senguelen = 766; Yindi = 755; Maferinyah Centre I = 660; Madinagbe = 608; Fandie = 608 and Moribayah = 565) over 25 days by manual aspiration from houses and human landing catches (HLCs). A subsample (n = 181) was selected for molecular form identification, of which 68% (123/181) and 31% (56/181) were determined to be *An. coluzzii* (M molecular form) and *An. gambiae*s.s. (S molecular form), respectively; two hybrid forms (*An. gambiae-An. coluzzii*) were also identified. While both species were sympatric across Forecariah Prefecture, proportions of *An. coluzzii* differed among study villages (Fisher's Exact test; $p = 0.007$), potentially attributable to varying local ecological factors[28] (Fig. 1A).

Insecticide resistance intensity. Levels of resistance to four insecticides (alpha-cypermethrin, bendiocarb, deltamethrin and permethrin) were assessed among 2229 female *An. gambiae* s.l. mosquitoes, collected across six study sites in Forecariah Prefecture (Fig. 1A,C). Local vectors were characterized by intense but highly variable pyrethroid resistance, with all populations demonstrating less than 90% mosquito mortality to the diagnostic doses (1X) of pyrethroids and most areas containing individuals capable of surviving exposure to 10X these insecticide concentrations (Figs 2 and 3). In addition, a subsample of mosquitoes still living following 30 minutes of insecticide exposure were held for up to two hours in treated bottles, with more than 30% of vectors capable of surviving this extended exposure time in Madinagbe (17/55), Moribayah (19/38), Senguelen (30/82) and Yindi (9/27); as compared to alpha-cypermethrin, deltamethrin had higher survival rates (prevalence ratio; PR = 1.60, CI = 1.01–2.57, $p = 0.049$) but permethrin did not (PR = 1.52, CI = 0.94–2.51, $p = 0.09$). When considering mosquitoes surviving two hours of pyrethroid exposure, compared to 1X, there was no evidence of lower survival at 2X of extended exposure (PR = 0.68, CI = 0.43–1.05, $p = 0.08$) or 5X (PR = 0.70, CI = 0.43–1.09, $p = 0.09$) but there was significantly lower survival at 10X (PR = 0.26, CI = 0.10–0.54, $p = 0.001$). The highest levels of pyrethroid resistance were observed in Moribayah, where mosquito mortality to two times the diagnostic doses of deltamethrin and permethrin was 38% and 32%, respectively (Fig. 2). Across all sites, levels of resistance of permethrin (PR = 6.67, CI = 2.41–23.76, $p = 0.001$) and deltamethrin (PR = 3.87, CI = 1.33–14.14, $p = 0.02$) were higher than alpha-cypermethrin.

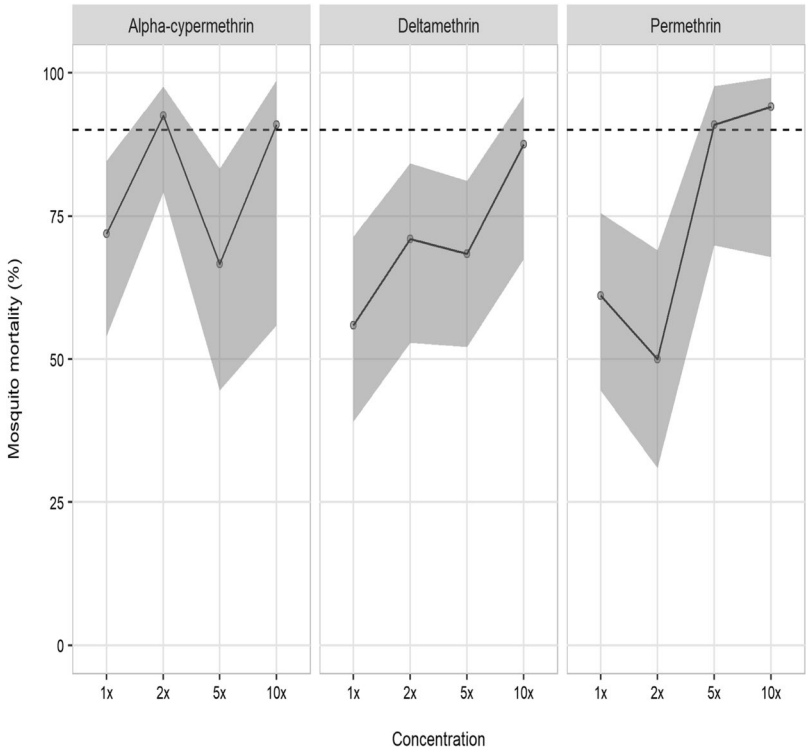

Figure 2. CDC resistance intensity assay data for three pyrethroid insecticides (alpha-cypermethrin, deltamethrin and permethrin) pooled across six study sites in Forecariah Prefecture (Fandie, Madinagbe, Maferinyah Centre I, Moribayah, Senguelen and Yindi). Estimates and confidence intervals are from a binomial regression model. Mortality below 90% (indicated by the dashed line) indicates the presence of resistance.

		% Mortality (numbers tested) after the diagnostic time			
Study Site	Insecticides	1X	2X	5X	10X
Fandie	Alpha-cypermethrin	79 (19)	86 (21)	83 (23)	89 (19)
	Alpha-cypermethrin + PBO	91 (22)	95 (19)	95 (22)	100 (13)
	Bendiocarb	100 (23)	-	-	-
	Deltamethrin	80 (20)	78 (22)	74 (19)	95 (20)
Madinagbe	Alpha-cypermethrin	79 (24)	80 (25)	88 (24)	88 (17)
	Bendiocarb	100 (19)	-	-	-
	Deltamethrin	78 (23)	89 (19)	89 (18)	86 (21)
	Permethrin	65 (20)	71 (21)	78 (27)	78 (18)
Maferinyah Centre I	Alpha-cypermethrin	85 (41)	98 (43)	94 (46)	87 (38)
	Bendiocarb	95 (40)	-	-	-
	Deltamethrin	83 (35)	89 (35)	83 (35)	97 (35)
	Permethrin	70 (20)	95 (20)	100 (21)	95 (20)
Moribayah	Alpha-cypermethrin	70 (20)	86 (21)	82 (22)	87 (23)
	Bendiocarb	95 (20)	-	-	-
	Deltamethrin	27 (22)	38 (21)	86 (22)	88 (16)
	Permethrin	25 (24)	32 (25)	86 (22)	95 (20)
Senguelen	Alpha-cypermethrin	85 (41)	88 (41)	92 (37)	93 (43)
	Bendiocarb	100 (22)	-	-	-
	Deltamethrin	76 (42)	77 (44)	86 (42)	83 (41)
	Deltamethrin + PBO	81 (16)	88 (16)	89 (19)	100 (11)
	Permethrin	38 (29)	40 (25)	96 (25)	100 (19)
Yindi	Alpha-cypermethrin	83 (42)	95 (43)	90 (30)	92 (25)
	Bendiocarb	100 (22)	-	-	-
	Deltamethrin	85 (40)	90 (39)	92 (36)	95 (42)
	Permethrin	73 (45)	89 (36)	92 (36)	100 (35)

Figure 3. Corrected percent mortality (and numbers tested) of *Anopheles gambiae* s.l. in CDC resistance intensity and synergist bioassays conducted in six sites in Forecariah Prefecture, Guinea.

Study Site	Survival Status	# Mosquitoes tested	Homozygote mutation (RR)	Heterozygote mutation (RS)	Homozygote wild type (SS)	N1575Y allele frequency		$\chi 2$ Test	*P* value
						R	*S*		
Fandie	Alive	51	0	8	43	0.08	0.92	0.37	0.543
	Dead	12	0	2	10	0.08	0.92	0.1	0.752
Madinagbe	Alive	35	0	3	32	0.04	0.96	0.07	0.791
	Dead	5	0	0	5	0	1.0	—	—
Maferinyah Centre I	Alive	43	0	10	33	0.12	0.88	0.74	0.390
	Dead	44	0	5	39	0.06	0.94	0.16	0.690
Moribayah	Alive	38	0	5	33	0.07	0.93	0.19	0.663
	Dead	0	0	0	0	—	—	—	—
Senguelen	Alive	73	1	7	65	0.06	0.94	2.14	0.144
	Dead	18	0	1	17	0.03	0.97	0.01	0.920
Yindi	Alive	35	0	3	32	0.04	0.96	0.07	0.791
	Dead	34	1	3	30	0.07	0.93	4.22	0.040

Table 1. N1575Y allele frequencies in *An. gambiae* s.l. from six study sites in Forecariah Prefecture, Guinea.

By comparison, levels of carbamate resistance were low, with complete mosquito susceptibility observed in Fandie, Madinagbe, Senguelen and Yindi; possible resistance (95% mosquito mortality) was restricted to two adjacent villages (Maferinyah Centre I and Moribayah) (Figs 1C and 2).

For all insecticides, there was no difference in ability to survive exposure between *An. coluzzii* (n = 123) and *An. gambiae* s.s. (n = 56) (PR = 0.85, CI = 0.70–1.02, $p = 0.09$ for *An. gambiae* s.s.) or association with survival and physiological status (i.e. blood-fed, unfed, gravid, etc.) (Fisher's Exact Test; $p = 1.00$).

Mosquito parity. Of 737 *An. gambiae* s.l. mosquitoes tested in resistance bioassays that had their ovaries inspected for parity, 46% were nulliparous (340/737), i.e. had not laid an egg batch, and 54% (397/737) were parous. There was no significant difference in proportion of parous mosquitoes by study village ($\chi^2 = 9.61$; $p = 0.09$) or by month tested ($\chi^2 = 2.18$; $p = 0.14$). However, for all three pyrethroids under evaluation, resistant mosquitoes were significantly more likely to be nulliparous (PR = 1.15, CI = 1.10–1.21, $p < 0.001$). This association was very similar across insecticides (alpha-cypermethrin: PR = 1.18, CI = 1.06–1.30, $p < 0.001$; deltamethrin: PR = 1.11, CI = 1.005–1.22, $p = 0.04$; and permethrin: PR = 1.18, CI = 1.05–1.33, $p = 0.002$). Small sample sizes precluded direct comparisons between pyrethroid resistance intensity and parity per insecticide. When considering resistance levels of all pyrethroids, nulliparous mosquitoes were more likely to be resistant to 2X (PR = 1.31, CI = 1.11–1.54, $p < 0.001$) and 10X (PR = 1.22, CI = 1.01–1.47, $p = 0.03$) but not to 5X (PR = 1.10, CI = 0.94–1.29, $p = 0.39$). Nulliparous mosquitoes were no more likely to be survivors of exposure to any dose of bendiocarb (1X: PR = 2.47, CI = 0.81–7.50, $p = 0.16$; 2X: PR = 0.50, CI = 0.11–2.32, $p = 0.70$; 5X: PR = 1.64, CI = 0.53–5.06, $p = 0.73$; 10X: PR = 1.63, CI = 0.59–4.49, $p = 0.66$).

Target site and metabolic mechanisms of resistance. N1575Y mutation screening was undertaken in a subset of 388 *An. gambiae* s.l. and was detected in 13% (49/388) of samples in both *An. coluzzii* (59%) and *An. gambiae* s.s. (41%); only two homozygote individuals were detected (Fig. 1B and Table 1). The frequency of the N1575Y resistant allele did not differ significantly among study districts (Fisher's Exact Test; $p = 0.71$) (Table 1 and Fig. 1B) (Table 1). There was no significant association between N1575Y allele frequency and ability of mosquitoes to survive pyrethroid exposure for 30 minutes (alpha-cypermethrin: PR = 0.85, CI = 0.63–1.13, $p = 0.42$; deltamethrin: PR = 1.06, CI = 0.81–1.37, $p = 0.94$; and permethrin: PR = 1.26, CI = 0.85–1.87, $p = 0.42$) or two hours (alpha-cypermethrin: PR = 0.26, CI = 0.03–2.04, $p = 0.32$; deltamethrin: PR = 0.64, CI = 0.19–2.14, $p = 0.76$; and permethrin: PR = 1.33, CI = 0.39–4.55, $p = 0.92$).

One hundred and twenty-eight base pairs upstream of N1575Y, a second non-synonymous mutation (I1527T) was identified among 10% of individuals (11/109); this mutation was not linked to N1575Y and only one individual from Maferinyah Centre I had both resistance mutations. All individuals with the I1527T resistant allele were heterozygous for this mutation and were identified as *An. gambiae* s.s. The frequency of the I1527T resistant allele did not vary among study villages (Fisher's Exact test; $p = 0.95$) (Table 2 and Fig. 1B). There was an association between I1527T allele frequency and ability of mosquitoes to survive pyrethroid exposure for 30 minutes for permethrin (PR = 2.80, CI = 1.03–7.64, $p = 0.04$), but not for the other two insecticides (alpha-cypermethrin: PR = 0.94, CI = 0.50–1.79, $p = 0.995$; and deltamethrin: PR = 1.08, CI = 0.52–2.20, $p = 0.99$). There were too few positive mosquitos to analyse associations at two hours.

The L1014F *kdr* allele was identified in 87% of samples (211/242), with the majority of mosquitoes presenting homozygous *kdr* profiles (83%; 200/242). The frequency of the L1014F *kdr* resistant allele varied significantly among study villages (Fisher's Exact test; $p = 0.0005$) (Table 3 and Fig. 1B). There were no associations between L1014F *kdr* allele frequency and ability of mosquitoes to survive insecticide exposure for 30 minutes (alpha-cypermethrin: PR = 0.86, CI = 0.49–1.49, $p = 0.88$; deltamethrin: PR = 1.59, CI = 0.995–2.55, $p = 0.054$; and permethrin: PR = 0.64, CI = 0.41–1.004, $p = 0.053$). There were not enough mosquitoes to assess associations between two hour survival and insecticide. When considering L1014F *kdr* and N1575Y as a single resistance haplotype, there was an association between presence of these mutations as compared to only the L1014F *kdr* mutation and ability of mosquitoes to survive permethrin exposure for 30 minutes (PR = 1.92,

Study Site	Survival Status	# Mosquitoes tested	Homozygote mutation (RR)	Heterozygote mutation (RS)	Homozygote wild type (SS)	I1527T allele frequency		$\chi 2$ Test	*P* value
						R	*S*		
Fandie	Alive	15	0	2	13	0.07	0.93	0.08	0.777
	Dead	0	0	0	0	—	—	—	—
Madinagbe	Alive	5	0	0	5	0	1.0	—	—
	Dead	0	0	0	0	—	—	—	—
Maferinyah Centre I	Alive	30	0	4	26	0.07	0.93	0.15	0.700
	Dead	19	0	2	17	0.05	0.95	0.06	0.806
Senguelen	Alive	17	0	1	16	0.03	0.93	0.02	0.888
	Dead	2	0	0	2	—	—	—	—
Yindi	Alive	16	0	2	14	0.06	0.94	0.07	0.791
	Dead	5	0	0	5	—	—	—	—

Table 2. I1527T allele frequencies in *An. gambiae* s.l. from five study sites in Forecariah Prefecture, Guinea.

Study Site	Survival Status	# Mosquitoes tested	Homozygote mutation (RR)	Heterozygote mutation (RS)	Homozygote wild type (SS)	L1014F *kdr* allele frequency		$\chi 2$ Test	*P* value
						R	*S*		
Fandie	Alive	29	29	0	0	1.0	0	—	—
	Dead	9	9	0	0	1.0	0	—	—
Madinagbe	Alive	26	8	3	15	0.63	0.37	14.67	<0.0001
	Dead	1	1	0	0	1.0	0	—	—
Maferinyah Centre I	Alive	41	40	0	1	0.98	0.02	41.00	<0.0001
	Dead	29	29	0	0	1.0	0	—	—
Moribayah	Alive	6	6	0	0	1.0	0	—	—
	Dead	0	0	0	0	—	—	—	—
Senguelen	Alive	30	28	0	2	0.93	0.07	30.00	<0.0001
	Dead	16	15	0	1	0.94	0.06	16.00	<0.0001
Yindi	Alive	32	21	6	5	0.75	0.25	8.00	0.005
	Dead	23	14	2	7	0.65	0.35	15.03	<0.0001

Table 3. L1014F *kdr* allele frequencies in *An. gambiae* s.l. from six study sites in Forecariah Prefecture, Guinea.

CI = 1.09–3.37, $p = 0.02$), but no association with alpha-cypermethrin (PR = 1.05, CI = 0.72–1.54, $p = 0.98$) or deltamethrin (PR = 1.11, CI = 0.78–1.58, $p = 0.87$). There was no association with survival data at two hours (alpha-cypermethrin: PR = 0.42, CI = 0.04–4.48, $p = 0.76$; deltamethrin: PR = 1.08, CI = 0.18–6.53, $p = 1.00$; and permethrin: PR = 1.58, CI = 0.33–7.50, $p = 0.86$).

The G119S *Ace-1* mutation was detected in 10% of samples (7/67) (Fig. 1C). All individuals with the resistant allele were heterozygous for this mutation and were identified as *An. gambiae* s.s. Frequencies of the G119S *Ace-1* mutation varied significantly among study districts (Fisher's Exact test; $p = 0.029$) (Table 4 and Fig. 1C). There was a significant association between presence of the G119S *Ace-1* mutation and ability of mosquitoes to survive 30 minute and two hour carbamate exposure (Fisher's Exact test; $p = 0.0047$ and Fisher's Exact test; $p = 0.0015$, respectively).

Across Forecariah Prefecture, significant deviations from Hardy-Weinberg equilibrium were observed for L1014F *kdr* in Madinagbe, Maferinyah Centre I, Senguelen and Yindi ($p < 0.005$ for all) (Table 3) and for N1575Y in Yindi ($p = 0.040$) but not for any other resistance loci.

Finally, to confirm the potential role of cytochrome P450 enzymes in pyrethroid resistance, mosquitoes collected in Fandie and Senguelen were pre-exposed to piperonyl butoxide (PBO), prior to intensity testing with alpha-cypermethrin and deltamethrin, respectively. In both populations, susceptibility was partially restored at five times the diagnostic dose of pyrethroids (95% and 89% mosquito mortality to alpha-cypermethrin and deltamethrin, respectively) and fully restored at 10X (Fig. 3).

Plasmodium falciparum infection and infectivity. The abdomens and head/thoraxes of 484 *An. gambiae* s.l. were screened separately to detect the presence of *Plasmodium falciparum* oocysts and sporozoites, respectively. Overall the proportion of positive mosquitoes for oocysts and sporozoites were 6.0% (29/484) and 0.2% (1/484), respectively. There was no significant difference in abdomen positive proportions among study villages (Fisher's Exact test; $p = 0.21$) or molecular forms (n = 179, 63% *An. coluzzii* and 37% *An. gambiae* s.s.; PR for *An. gambiae* s.s. = 1.32, CI = 0.28–5.24, $p = 0.70$). In mosquitoes that survived insecticide exposure, oocyst rate was 6.8% (23/340) compared to 4.2% (6/144) in their susceptible counterparts (PR = 1.62, CI = 0.72–4.26, $p = 0.28$). The single sporozoite positive individual was collected from Maferinyah Centre I and died following 2X alpha-cypermethrin exposure.

Study Site	Survival Status	# Mosquitoes tested	Homozygote mutation (RR)	Heterozygote mutation (RS)	Homozygote wild type (SS)	G119S *Ace-1* allele frequency		$\chi 2$ Test	*P* value
						R	*S*		
Fandie	Alive	9	0	1	8	0.06	0.94	0.03	0.862
	Dead	7	0	0	7	0	1.0	—	—
Madinagbe	Alive	3	0	1	2	0.17	0.83	0.12	0.729
	Dead	1	0	0	1	0	1.0	—	—
Maferinyah Centre I	Alive	17	0	2	15	0.06	0.94	0.07	0.791
	Dead	11	0	1	10	0.05	0.95	0.02	0.888
Moribayah	Alive	2	0	2	0	0.5	0.5	2.00	0.157
	Dead	—	—	—	—	—	—	—	—
Senguelen	Alive	—	—	—	—	—	—	—	—
	Dead	9	0	0	9	0.0	1.0	—	—
Yindi	Alive	—	—	—	—	—	—	—	—
	Dead	8	0	0	8	0.0	1.0	—	—

Table 4. G119S *Ace-1* allele frequencies in *An. gambiae* s.l. from six study sites in Forecariah Prefecture, Guinea.

Considering the interaction between pyrethroid resistance and *P. falciparum* infection, there was no significant relationship between abdomen positivity rate and survival following insecticide exposure in all pooled data (PR = 1.36, CI = 0.48–5.52, $p = 0.61$) or per insecticide (alpha-cypermethrin: Fisher's Exact test; $p = 0.65$; deltamethrin: Fisher's Exact test; $p = 0.57$ and permethrin: Fisher's Exact test; $p = 0.20$). Considering the interaction between carbamate resistance and *P. falciparum* infection, survivors of bendiocarb exposure were significantly more likely to be infected (Fisher's Exact test; $p = 0.049$; 4/23 survivors *vs.* 3/76 susceptible individuals).

No significant associations were observed between presence of target site mutations and abdomen positivity (N1575Y: Fisher's Exact test, $p = 0.76$, I1527T: Fisher's Exact test, $p = 1.00$, L1014F *kdr*: Fisher's Exact test, $p = 0.37$, and G119S *Ace-1*: Fisher's Exact test, $p = 0.50$).

Discussion

By 2016, resistance to at least one insecticide has been reported from over 80% of malaria endemic countries[1], representing a threat to the continued efficacy of key malaria control strategies. However, the relative impact of decreased mosquito susceptibility on vectorial capacity remains unknown, largely due to a paucity of field data. To begin to address this deficit, in an area of high malaria transmission in Guinea, we characterized levels of insecticide resistance and age of local vector populations, in combination with molecular identification of resistance markers and detection of malaria infection.

In Forecariah Prefecture, intense pyrethroid resistance was abundant, evidenced by vector populations that were not only resistant to ten times the insecticide concentration required to kill susceptible individuals, but were also capable of surviving these doses for up to two hours. These observations are of concern given that pyrethroid LLINs are the only malaria vector control intervention deployed in this area. The restoration of mosquito susceptibility following pre-exposure to PBO and the association of mutations in the voltage-gated sodium channel (VGSC; L1014F *kdr*-N1575Y haplotype and I1527T) with lower mortality to permethrin suggests that both target site mutations and increased activity of P450 monooxygenases are contributing to pyrethroid resistance. This finding aligns with reports from Burkina Faso where upregulated detoxification enzymes were responsible for extreme pyrethroid resistance in *An. coluzzii*, with N1575Y associated with more limited tolerance to deltamethrin[29]. It has been proposed that N1575Y may compensate for fitness costs incurred by the L1014F *kdr* mutation and provide additional pyrethroid resistance[30]. The I1527T mutation is located within the III S6 helix of the VGSC, adjacent to a predicted pyrethroid/DDT binding site; nearby residues have already been implicated in resistance in other medically-important vector species[31,32]. However, to date, a role for I1527T in phenotypic resistance in *An. gambiae* s.l. has not been confirmed, nor has this mutation been detected in Guinea previously.

Of the three pyrethroid insecticides evaluated, resistance levels were often highest to permethrin, despite all mass LLIN campaigns in the country having exclusively distributed deltamethrin-treated products[25]. This may be explained by L1014F *kdr* playing a larger contributing role to resistance to type I (permethrin) versus type II (alpha-cypermethrin and deltamethrin) pyrethroids[33]. Levels of bendiocarb resistance were comparatively lower, which was not unexpected considering IRS is not routinely conducted by the Guinean NMCP. In the case of carbamate resistance, the presence of the G119S *Ace-1* mutation was highly predictive of bioassay survivorship and tolerance to increased exposure times. Previous studies have demonstrated that the G119S *Ace-1* substitution imposes a high fitness cost[34,35] by decreasing affinity of the resistant enzyme for its substrate by more than 60%[36]; heterogeneous or homogeneous duplication of this locus has been proposed to restore activity[37]. In Forecariah Prefecture, frequency of the G119S *Ace-1* resistant allele (of undetermined copy number) was low and could be a result of selective pressure imposed by unregulated agricultural use of carbamates and organophosphates, which were readily available at the local market (S. Irish, personal communication).

Our results demonstrated that pyrethroid resistance was not associated with malaria prevalence in the mosquitoes that were tested, with no significant differences observed between *Plasmodium* infection in susceptible or resistant individuals. Our findings contrast with a number of laboratory studies ascribing potential fitness costs to insecticide resistance which may have the collateral benefit of reducing malaria transmission[16,18–22]. However,

in our study, survivors of bendiocarb exposure were significantly more likely to be infected with *P. falciparum*. Pyrethroid exposure has previously been shown to adversely affect *P. falciparum* development in L1014F *kdr* resistant *An. gambiae* s.s. in Uganda[21], which may explain part of our data, if we assume resistant individuals are able to survive contact with LLINs and susceptible vectors are not. Alternative observations have also been reported whereby *kdr* has been shown to potentiate the vector competence of *An. gambiae* s.s.[23], and *Plasmodium* infection has partially restored DDT susceptibility in *An. gambiae* carrying the *kdr* mutation[38] but also reduced the survival of resistant vectors in the absence of insecticide exposure[17]. The discrepancies between *in vitro* studies and our field data may reflect more generalised fitness variation between laboratory mosquito strains and wild populations and/or different underlying resistance mechanisms, e.g. target site mutations *vs*. increased activity of metabolic enzymes, and strongly support the need for additional studies in areas of differing resistance mechanisms and disease transmission intensities.

We examined the impact of resistance on mosquito age as increasing age has been proposed to restore insecticide susceptibility[39–46] and insecticide exposure may still reduce resistant vector life-span through delayed mortality effects[16]. In Forecariah Prefecture, we observed a similar phenomenon, with resistant mosquitoes having significantly lower parity rates than their susceptible counterparts.

Elucidating the interaction between insecticide resistance and vectorial capacity is complex and challenging in field conditions and a number of limitations were encountered during this study. Adult female mosquitoes were sampled using HLCs and via manual aspiration from house walls to maximise the number of individuals available for bioassay testing. However, these strategies may have introduced a bias in species composition collected; previous studies have suggested that proportions of *An. gambiae* s.s. and *An. coluzzii* may differ significantly between larval and adult spray catches[47]. We also encountered issues with individuals that did not amplify with two sub-species PCR assays[48,49], potentially reflecting polymorphisms at the primer binding sites and/or the presence of a cryptic sub-species; previously a cryptic subgroup GOUNDRY has been reported, with close genetic affinities to *An. coluzzii* and enhanced susceptibility to *P. falciparum* infection[50–52]. While use of F_1 progeny mosquitoes is recommended for resistance testing[53,54], to investigate the impact of resistance on *Plasmodium* infection in the vector, it was necessary to sample wild caught adults of undetermined, mixed age and physiological status; the latter parameter may have varied between sampling methods, with more fed mosquitoes collected from inside houses, compared to unfed, host-seeking mosquitoes in HLCs. As a consequence, mortality in our study may have been under-estimated, considering the proposed inverse relationship between vector resistance and age[39–45]. Similarly, blood-feeding among resistant mosquitoes has been suggested to increase insecticide tolerance[55], however, no association between physiological status and resistance was observed in our dataset. Our study would have also benefitted from larger sample sizes of mosquitoes tested in bioassays, to statistically power comparisons between different resistance intensities. Furthermore, abdomen positive mosquitoes cannot all be assumed to become infective sporozoite-transmitters, particularly if vector life-span is reduced; a positive abdomen can also reflect a recent feed on an infected individual or the passage of sporozoites to the salivary glands. Finally, manual parity dissections have a number of known constraints, including insensitivity, especially in low endemicity areas, and inter-operator subjectivity. While every effort was made to consistently bisect individuals, and our reported sporozoite rate was low, other studies have demonstrated higher number of sporozoite false positives by PCR when abdomens were removed posterior to the junction of the abdomen and thorax[56]. We also cannot entirely discount natural variation in parity, which may have increased over the sampling period at the beginning of the rainy season.

Conclusions

These findings present a comprehensive overview of the current levels of insecticide resistance and underlying target site mutations present in Maferinyah, Guinea, an area of high malaria transmission. Local mosquito populations were intensely resistant to pyrethroids (alpha-cypermethrin, deltamethrin and permethrin), associated with high frequencies of the L1014F *kdr* allele. N1575Y and I1527T mutations in the VGSC gene were present at lower levels and may warrant increased surveillance efforts, particularly as L1014F *kdr* approaches fixation. Restoration of mosquito susceptibility following pre-exposure to PBO indicates increased activity of detoxification enzymes is also contributing to pyrethroid resistance in this area and requires additional characterization. Despite no ongoing vector control activities using carbamates, bendiocarb resistance was observed, and the G119S *Ace-1* allele was detected in a subset of tolerant individuals. Malaria infection was not associated with pyrethroid resistance but it was associated with bendiocarb resistance. In general, resistant vectors were younger than their susceptible counterparts. Further studies are necessary to investigate the impact of insecticide resistance on vector fitness, including mosquito fecundity, egg viability, hatchability and parasite development following an infected blood meal.

Methods

Study area and mosquito collections. Mosquito collections were undertaken in six villages in the Maferinyah sub-prefecture, Forecariah Prefecture (Fandie, Madinagbe, Maferinyah Centre I, Moribayah, Senguelen and Yindi), in Southwest Guinea. Deltamethrin-treated LLINs had been distributed as part of a national mass campaign in Maferinyah in 2016. Sampling was conducted between 22nd June and 17th July 2017, coinciding with the beginning of the long rainy season. Following consent from the household owner, indoor resting, female *Anopheles* mosquitoes were collected from house walls by manual aspiration between 7:00 h and 12:00 h. In the same villages, mosquitoes were also sampled using HLCs, carried out between 22:00 h and 3:00 h. Mosquitoes were stored in cages with access to 10% sugar solution, prior to transport to the Centre de Formation et de Recherche en Santé rurale de Maferinyah (CNFRSR) for analysis.

CDC resistance intensity and synergist bioassays. All bioassays were performed using mosquitoes identified morphologically as *An. gambiae* s.l.[57]; wild caught females were held for a maximum of 48 hours before testing. Centers for Disease Control and Prevention (CDC) resistance intensity bioassays for three pyrethroid insecticides (alpha-cypermethrin, deltamethrin and permethrin) were conducted according to published guidelines[53]. Stock solutions of 1, 2, 5 and 10 times the diagnostic dose of insecticide (alpha-cypermethrin: 12.5 μg/bottle; deltamethrin: 12.5 μg/bottle; and permethrin: 21.5 μg/bottle), were prepared by diluting technical grade insecticide in 50 ml of acetone. Bioassays for bendiocarb were conducted using the diagnostic dose (1X: 12.5 μg/bottle). The inside of each Wheaton 250 ml bottle along with its cap was coated with 1 ml of stock solution by rolling and inverting the bottles. In each test, a control bottle was coated with 1 ml of acetone. Following coating, bottles were left to dry in a dark box. Approximately 15–25 field-caught adult female *An. gambiae* s.l. of unknown age and mixed physiological status were introduced into each bottle using a mouth aspirator and mortality was recorded at 15 minute intervals until all were dead or up to two hours; dead individuals at 30 minutes were removed from bottles by aspiration and again after two hours. In each bioassay, a control bottle, coated with acetone, was run in parallel. Mortality in control bottles never exceeded 5%. In select sites with significant pyrethroid resistance, synergist assays were also conducted by pre-exposing mosquitoes to piperonyl butoxide (PBO) for 1 hour (100 μg/bottle) prior to performing pyrethroid bioassays. Multiple replicates were performed per insecticide and study village, depending on mosquito availability, and individual surviving (resistant) and dead (susceptible) mosquitoes were preserved in RNAlater® (Thermo Fisher Scientific, UK) at −20 °C at the London School of Hygiene and Tropical Medicine (LSHTM) and CDC. Prior to molecular analysis, mosquito heads/thoraxes were separated from abdomens under a dissecting microscope and stored separately.

Parity dissection. Ovarian dissection to determine mosquito parity was performed on ten mosquitoes (including both survivors and those that died) selected randomly from each bottle after bioassay completion[58]. The ovaries of each mosquito were dissected on a sterile microscope slide in distilled water, using a binocular dissection microscope and physiological status was determined. The ovaries were then examined under a light microscope (10X magnification) for the presence of tightly coiled skeins or loose coils, indicative of a nulliparous or parous ovary, respectively. On completion of ovarian dissection, head/thoraxes and abdomens of each mosquito were stored separately in RNAlater® (Thermo Fisher Scientific, UK) at −20 °C.

Molecular species identification. A subset of susceptible and resistant *An. gambiae* s.l. mosquitoes from all six villages containing both nulliparous and parous individuals were selected for molecular analysis at the LSHTM and CDC. Genomic DNA from dissected body parts was extracted per protocol using Qiagen DNeasy 96 Blood and Tissue kits (Qiagen, UK) at LSHTM or Extracta™ DNA Prep for PCR-Tissue kits (QuantaBio, USA) at CDC.

At LSHTM, molecular species identification was performed using a multiplex TaqMan real time PCR assay to detect and discriminate *An. gambiae* and *An. arabiensis*[59]. PCR reactions were prepared using Qiagen Quantitect Probes Master mix (Qiagen) with each reaction containing 6.25 μl of master mix, a final concentration of 0.8 μM of each primer, 0.2 μM of probe *An. arabiensis* (Cy5), 80 nM of probe *An. gambiae* (FAM) and 1 μl of template DNA for a final reaction volume of 12.5 μl. Prepared reactions were run on a Stratagene Mx30005P QPCR system for 10 min at 95 °C, followed by 40 cycles of 95 °C for 25 sec and 66 °C for 60 sec. The increases in the species-specific FAM and Cy5 fluorophores were detected in real-time at the end of each cycle and results were analysed using Stratagene MxPro QPCR software. Positive controls from gDNA extracted from known *An. gambiae* and *An. arabiensis* individuals were included in each run, in addition to no template controls (NTCs). Sub-samples of individuals from each district confirmed as *An. gambiae* were further distinguished as *An. coluzzii* or *An. gambiae* s.s by targeting a *SINE200* insertion only present in *An. coluzzii*[48]. PCR reactions were prepared using Hot Start *Taq* 2X Master Mix (New England Biolabs, UK) with each reaction containing 12.5 μl of master mix, a final concentration of 1 μM of each primer, 2 μl template DNA for a final reaction volume of 25 μl. Prepared reactions were amplified using a BIORAD T100 Thermal Cycler for 10 min at 95 °C, followed by 35 cycles of 94 °C for 30 sec, 54 °C for 30 sec, 72 °C for 1 min and a final extension of 72 °C for 10 min. PCR products were separated and visualised using 2% Egel EX agarose gels (Invitrogen, UK) with SYBR safe and an Invitrogen E-gel iBase Real-Time Transilluminator. *An. coluzzii* individuals with the insertion resulted in a single PCR product of 479 bp and *An. gambiae* s.s. a PCR product of 249 bp.

At CDC, *An. coluzzii* and *An. gambiae* s.s. specimens were differentiated by targeting single nucleotide polymorphisms (SNPs) present in rDNA[49]. PCR reactions were prepared using 20–40 ng of DNA, 5X Green GoTaq® Reaction Buffer (Promega, USA), 25 mM $MgCl_2$, 2.5 mM of each dNTP, 1 U GoTaq® DNA polymerase and 25 pmol/μl of primers IMP-UN, AR-3T, GA-3T, IMP-S1 and IMP-M1 in a final volume of 25 μl. PCR reaction conditions were 95 °C for 5 min, followed by 30 amplification cycles (95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec) and a final elongation step at 72 °C for 5 min. Amplified PCR products were visualized on 1.5% agarose gels, stained with GelRed™ (Biotium, USA). *An. arabiensis* Dongola, *An. coluzzii* AKDR and *An. gambiae* s.s. RSP-ST strains from the Malaria Research and Reference Reagent Resource Center (MR4), were used as positive controls. Amplification products of 463 bp and 333 bp or 463 bp and 221 bp were indicative of *An. coluzzii* or *An. gambiae* s.s., respectively.

Insecticide target site mutation detection. Detection of the L1014F West African *kdr* mutation was performed on a subset of individuals from all six districts according to the adapted protocol for allele-specific PCR developed by Martinez-Torres *et al.*[60]. At both LSHTM and CDC, PCR reactions were prepared using Hot Start *Taq* 2X Master Mix (New England Biolabs) with each reaction containing 12.5 μl of master mix and variable final concentrations of primers (IPCF 0.1 μM, AltRev 0.1 μM, West WT 1 μM, West West 1.1 μM) for a final reaction volume of 25 μl. Prepared reactions were run on a BIO-RAD QPCR system for 5 min at 95 °C, followed by 35

cycles of 95˚C for 30 sec, 59˚C for 30 sec and 72˚C for 30 sec and a final extension of 72˚C for 5 min. PCR products were separated and visualised using 2% Egel EX agarose gels (Invitrogen) with SYBR safe and an Invitrogen E-gel iBase Real-Time Transilluminator. A PCR product of 214 bp indicated the susceptible wild type allele and a PCR product of 156 bp indicated the resistant allele. *An. coluzzii* AKDR and *An. gambiae* s.s. RSP-ST were used as positive and negative controls for L1014F *kdr*, respectively.

At LSHTM, a larger sub-sample of individuals from all six districts was chosen to be screened for the N1575Y mutation (previously shown to have low prevalence in West African populations) using the TaqMan real time PCR assay developed by Jones *et al.*[30]. PCR reactions were prepared using Qiagen Quantitect Probes Master mix (Qiagen) with each reaction containing 10 µl of master mix, a final concentration of 1 µM of each primer and 0.5 µM of each probe, 5 µl of PCR grade water and 2 µl of template DNA, for a final reaction volume of 20 µl. Prepared reactions were run on a Roche LightCycler 96 System for 15 min at 95˚C, followed by 35 cycles of 94˚C for 15 sec and 60˚C for 60 sec. Positive controls from gDNA extracted from known *An. gambiae* s.s. with or without the N1575Y mutation were included on each run in addition to no template controls (NTCs). PCR results were analysed using the LightCycler 96 software (Roche Diagnostics).

At CDC, to detect the N1575Y mutation, a 218 bp fragment of the VGSC channel spanning domains III-IV was sequenced. PCR reactions were prepared using 25 µl of 2X AccuStart™ II PCR SuperMix, a final concentration of 20 pmol/µl of primers Exon_29_F (5′-AAATGCTCAGGTCGGTAAACA-3′) and Exon_29_R (5′-GCCACTGGAAAGAATGGAAA-3′)[30] and 2 µl of template DNA, for a final reaction volume of 50 µl. PCR reaction conditions were 95 °C for 3 min, followed by 35 amplification cycles (95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec) and a final elongation step at 72 °C for 5 min. Amplified PCR products were visualized on 2% agarose gels, stained with GelRed™ (Biotium, USA) and purified using the 96-well Millipore™ MultiScreen™ HTS vacuum manifold system. Bi-directional sequencing was performed using primers Exon_29_F and Exon_29_R with the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's protocol. Sequencing reactions were purified using the Big Dye® Xterminator™ Purification Kit (Applied Biosystems, USA), according to the manufacturer's protocol and data were generated using a 3500xL Genetic Analyzer (Applied Biosystems, USA). Sequences were assembled manually in BioEdit v7.0.9.0 sequence alignment editor software (Ibis Biosciences, USA) and unambiguous consensus sequences were produced for each individual. Consensus sequences are available from GenBank under the accession numbers MH929325 - MH929433.

The presence of the G119S *Ace-1* mutation was determined using PCR restriction fragment length polymorphism analysis[61]. At both LSHTM and CDC, PCR reactions were prepared using Hot Start *Taq* 2X Master Mix (New England Biolabs) with each reaction containing 12.5 µl of master mix, a final concentration of 1 µM of each primer, 2 µl template DNA, to a final reaction volume of 25 µl. Prepared reactions were run on a BIO-RAD QPCR system for 5 min at 95˚C, followed by 30 cycles of 95˚C for 30 sec, 52˚C for 30 sec and 72˚C for 1 min and a final extension of 72˚C for 5 min. PCR products were digested using the *Alu*I restriction enzyme through incubation at 37˚C for 16 hr followed by 65˚C for 20 min. DNA fragments were visualised on 2% Egel EX agarose gels (Invitrogen) with SYBR safe and an Invitrogen E-gel iBase Real-Time Transilluminator. 194 bp undigested PCR products indicated the susceptible allele and 74 bp and 120 bp digested fragments indicated the presence of the resistant allele. Presence of all three product sizes indicated that the sample was heterozygous.

Plasmodium falciparum detection. Bisected mosquito head/thoraxes and abdomens from bioassay individuals were screened separately for *P. falciparum* infection, by targeting a 120 bp sequence of the *P. falciparum* cytochrome c oxidase subunit 1 (*cox1*) mitochondrial gene[62]. At LSHTM real-time PCR reactions were prepared using FastStart SYBR Green Master mix (Roche Diagnostics) with each reaction containing 5 µl of master mix, a final concentration of 1 µM of each primer, 1 µl of PCR grade water and 2 µl template DNA, to a final reaction volume of 10 µl. Prepared reactions were run on a Roche LightCycler 96 System for 15 min at 95˚C, followed by 35 cycles of 95˚C for 15 sec and 58˚C for 30 sec. Amplification was followed by a dissociation curve (95˚C for 10 sec, 65˚C for 60 sec and 97˚C for 1 sec) to ensure the correct target sequence was amplified. Positive controls from gDNA extracted from a cultured *P. falciparum*-infected blood sample (parasitaemia of ~10%) were included on each run, in addition to no template controls (NTCs). PCR results were analysed using the LightCycler® 96 software (Roche Diagnostics).

At CDC, a conventional PCR was used for *cox1* detection[63]. PCR reactions were prepared using 6.25 µl of2X AccuStart™ II PCR SuperMix, a final concentration of 5 µM of primers COX-IF (5′-AGAACGAACGCTTTTAACGCCTG-3′) and COX-IR (5′-ACWGGATGGACTTTATATCCACCATTAAGT-3′)[63] and 2 µl of template DNA, for a final reaction volume of 12.5 µl. PCR reaction conditions were 94 °C for 5 min, followed by 40 amplification cycles (94 °C for 1 min, 62 °C for 1 min, 72 °C for 1.5 min) and a final elongation step at 72 °C for 10 min. Amplified PCR products were visualized on 2% agarose gels, stained with GelRed™ (Biotium, USA), alongside the same positive and negative controls used in the real-time assay. A positive head/thorax or abdomen was assumed to indicate the presence of sporozoites or oocysts, respectively.

Data analysis. Percent mosquito mortality for each insecticide dose was interpreted using the WHO criteria: 98–100% mortality at 30 minutes of exposure indicates 'susceptibility', 90–97% mortality suggests 'possible resistance' and <90% indicates the presence of 'resistance'[54]. Where molecular species ID data were available, results are reported for *An. coluzzii* and *An. gambiae* s.s. separately; otherwise individuals are referred to as *An. gambiae* s.l. Pearson's Chi squared tests and Fisher's exact tests (when sample sizes were small) were used to investigate the statistical association between survival by site, allele frequencies, and deviations from Hardy-Weinberg equilibrium. Other analyses utilized quasi-Poisson regression to estimate prevalence ratios (PRs) of mosquito survival, except the model in Fig. 2, which used a binomial model to ensure confidence intervals did not extend beyond 100%. Outcomes from pyrethroid bioassays were evaluated separately to carbamate assays, unless otherwise

specified. The endpoint of all bioassays was considered to be 30 minutes (the diagnostic time for the insecticides under evaluation), unless otherwise specified. All statistical analyses were performed in R version 3.5.1[64], with the level of significance set at $\alpha = 0.05$.

Ethical approval and consent to participate. The study protocol was reviewed and approved by the Comite National d'Ethique pour la Recherche en Sante (030/CNERS/17) and the institutional review boards (IRB) of the London School of Hygiene and Tropical Medicine (#13612 and #14076) and the Centers for Disease Control and Prevention, USA (2018–086); all study procedures were performed in accordance with relevant guidelines and regulations. Prior to study initiation, community consent was sought from village leaders and written, informed consent was obtained from the heads of all households selected for participation and from all fieldworkers who performed HLCs. Study information was provided to participants in French, *Susu*, *Foula* and *Malinké*. Fieldworkers participating in human landing catches were provided with doxycycline malaria prophylaxis for the duration of the study.

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Author Contributions

L.A.M., S.I., T.W., M.S. and A.H.B. designed the study and were responsible for data analysis and interpretation. E.C., N.M.V. and M.S. led the entomology field activities and participated in data collection. E.C., N.V.M., T.W., J.O., L.A.M. and G.L. performed the molecular assays. R.E.W. provided statistical expertise and data analysis support. L.A.M., T.W., S.I., E.C. and N.M.V. drafted the manuscript which was revised by co-authors. All authors read and approved the final manuscript.

Additional Information

Competing Interests: The authors declare no competing interests

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