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## Insecticide Resistance Allele Frequencies in *Anopheles gambiae* before and after Anti-Vector Interventions in Continental Equatorial Guinea

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**Abstract.** Anti-malaria interventions that rely on insecticides can be compromised by insecticide-resistance alleles among malaria vectors. We examined frequency changes of resistance alleles at two loci, knockdown resistance (*knr*) and acetylcholinesterase-1 (*ace-1*), which confer resistance to pyrethroids and DDT, and carbamates, respectively. A total of 7,059 *Anopheles gambiae* sensu stricto mosquitoes were analyzed from multiple sites across continental Equatorial Guinea. A subset of sites included samples collected pre-intervention (2007) and post-intervention (2009–2011). Both L1014S and L1014F resistance alleles were observed in almost all pre-intervention collections. In particular, L1014F was already at substantial frequencies in M form populations (17.6–74.6%), and at high frequencies (> 50%) in all but two S form populations. Comparison before and throughout anti-vector interventions showed drastic increases in L1014F, presumably caused by intensified selection pressure imposed by pyrethroids used in vector control efforts. In light of these findings, inclusion of other insecticide classes in any anti-vector intervention can be considered prudent.

### INTRODUCTION

Insecticide resistance poses a significant threat to the success of anti-malaria interventions that rely on indoor residual spraying (IRS) and insecticide-treated nets (ITNs). Resistance in the major Afrotropical malaria vector *Anopheles gambiae* to pyrethroids, DDT, and carbamate class insecticides includes target site resistance in specific genes that changes the sensitivity of the carrier to insecticides.<sup>1–3</sup> DDT and pyrethroids insecticides target the voltage-gated sodium channel, and molecular characterizations have shown that various mutations in the S1–S6 trans-membrane segments of domain II of this gene provides resistance to these insecticides in a number of insect species while preserving voltage-gated sodium channel function in the presence of pyrethroid-class and DDT insecticides.<sup>4,5</sup> In *An. gambiae* sensu stricto, two point mutations are present at amino acid position 1014 of the gene, both of which result in an amino acid substitution conferring reduced susceptibility to pyrethroids and DDT.

The L1014F mutation was first documented in *An. gambiae* sensu stricto mosquitoes from Côte d'Ivoire in 1998.<sup>1</sup> Soon after, the L1014S mutation, was discovered in *An. gambiae* collected in Kenya.<sup>2</sup> Subsequently, these two alleles became nominally associated with the regions where they were first detected and were called knockdown resistance-w (*knr-w*) (L1014F) and *knr-e* (L1014S), respectively. The L1014F allele results from a substitution of the leucine residue at that position with phenylalanine, whereas the L1014S allele is the result of a replacement with serine.<sup>1,2</sup> Subsequent studies have shown that these mutations are not geographically restricted, but can be found within *An. gambiae*, as well as *An. arabiensis* populations.<sup>6–9</sup> The L1014F allele is present in *An. gambiae* sensu lato mosquitoes as far east as Uganda

and Ethiopia and conversely, the L1014S allele has been detected throughout west and central Africa.<sup>7,9–15</sup>

As reliance on pyrethroids and DDT for vector control operations has increased, *knr* allele frequencies have risen across Africa, often to high levels. For example, Stump and others reported significant increases in *knr* allele frequencies among *An. gambiae* collected from villages in western Kenya that received trial permethrin-treated bed net interventions.<sup>16</sup> L1014S frequencies doubled from approximately 3–4% in *An. gambiae* mosquitoes collected in 1987 to approximately 8% in 2001 and 2002 among villages that received treated nets, whereas frequencies of the allele remained the same in non-intervention sites. In another study, Mathias and others evaluated spatio-temporal variations in *knr* frequency in *An. gambiae* in the same two villages during 1996–2010, during which pyrethroid ITN interventions were dramatically scaled up. They reported “a sharp increase in homozygous frequencies from complete absence in both locations initially to 80.5% in Seme in 2008 and 91.7% for Asembo in 2010.”<sup>17</sup> In a recent study on the impact of a large scale IRS and ITN campaign in southern Benin, *knr* frequencies were also found to have increased, although this was also true for areas in which no planned interventions were implemented, underscoring the effect of the agricultural and household insecticide use on resistance.<sup>18</sup>

In east Africa, a similar increase has been observed. For example, in Uganda where DDT is used for IRS and deltamethrin-impregnated ITNs are the cornerstone of vector suppression efforts, a significant increase in L1014S frequencies was observed in *An. gambiae* sensu stricto in three out five sites during 2001–2002 and 2004–2006.<sup>19</sup> In Ethiopia, L1014F allele frequencies in excess of 98% were found in *An. arabiensis* exposed to DDT, which was used for IRS and ITN control measures.<sup>20</sup> In a follow-up study after DDT was discontinued in favor of deltamethrin, > 96% of *An. arabiensis* vectors were determined to be homozygous and 3.6% were found to be heterozygous for the L1014F allele.<sup>20</sup> These studies demonstrate that where pyrethroids

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and DDT have been applied intensively as part of anti-vector interventions, selection of *kdr* alleles soon follows. As such, vigilant monitoring of resistance is requisite to inform operational decisions regarding pyrethroid and DDT use as anti-malaria efforts continue to scale up across Africa.

*Anopheles gambiae* sensu stricto is subdivided into two molecular forms, the M and the S form, that are widely considered to be incipient species.<sup>21</sup> The *kdr* allele originated in the S molecular form of *An. gambiae*, and is at lower frequency in the M molecular form of *An. gambiae* in most locations. Nonetheless, an increase in *kdr* allele frequencies was also observed in *An. gambiae* M molecular forms in which *kdr* frequencies increased from 0.5% before initiation of a nationwide ITN distribution program in Niger to 7.2% two seasons later.<sup>22</sup>

To date, resistance mutations at the same location in the sodium channel gene have not been reported in *An. funestus*, another important malaria vector distributed throughout sub-Saharan Africa.<sup>23</sup> However, a recent report of pyrethroid resistance in field-caught *An. funestus* mosquitoes from Uganda, concluded that although neither *kdr* allele was detected, a "correlation between haplotypes and resistance phenotype was observed indicating that mutations in other exons may be conferring the knockdown resistance in this species."<sup>24</sup> Knockdown alleles have been documented beyond Africa in species such *An. stephensi* and *An. culicifacies*, important malaria vectors throughout southern Asia and the Middle East.<sup>25–27</sup>

As *kdr* allele frequencies in *An. gambiae* populations have increased throughout Africa, IRS programs have sometimes switched to alternative insecticide classes such as carbamates and organophosphates. As such, the targets of carbamates and other insecticides have also been under heavy selection.<sup>28</sup> One such target is the acetylcholinesterase-1 (*ace-1<sup>R</sup>*), which serves an essential role in neurotransmission in many arthropods and is targeted by several classes of insecticides, including carbamates and organophosphates.<sup>29,30</sup> Target site resistance in this gene, the *ace-1<sup>R</sup>* allele in *An. gambiae*, is conferred through a single amino acid substitution from glycine to serine at residue 119.<sup>3</sup> The *ace-1<sup>R</sup>* alleles are becoming increasingly prevalent in *An. gambiae* populations in west Africa.<sup>28,31</sup> Reduced susceptibility to carbamate insecticides has been described through the use of bioassays and molecular detection of the *ace-1<sup>R</sup>* allele in *An. gambiae* M and S populations in Côte d'Ivoire, southern Benin, and Burkina Faso.<sup>32–36</sup> Similar to the distribution of *kdr* alleles in continental west Africa, the *ace-1<sup>R</sup>* allele was far more prevalent in the S (32%) than the M (3.6%) molecular form.<sup>36</sup> In Côte d'Ivoire, *ace-1<sup>R</sup>* allele frequencies have reached levels as high as 30.9% and 35.2% in M and S molecular forms, respectively.<sup>34</sup> The geographic distribution of *ace-1<sup>R</sup>* alleles appears to be closely correlated with areas in which carbamate insecticides are applied as part of agricultural pest management, particularly in cotton and vegetable-growing areas in west Africa.<sup>35,36</sup>

In 2007, the Equatorial Guinea Malaria Control Initiative (EGMCI), a comprehensive anti-malaria program with a strong emphasis on anti-vector interventions, was initiated in continental Equatorial Guinea. Such interventions included indoor residual spraying of pyrethroids and carbamates in two provinces (Litoral and Kie N'tem) and distribution of ITNs containing deltamethrin in two other provinces (Centro

Sur and Wele Nzaz). The EGMCI also included monitoring of vector abundance, sporozoite rates, and insecticide-resistance alleles at multiple sites across continental Equatorial Guinea to inform operational decisions on vector suppression efforts.

A study performed in 2004–2005 provided the first report of the presence of the two resistance alleles (L1014S and L1014F) in *An. gambiae* sensu stricto populations in continental Equatorial Guinea.<sup>13</sup> This study surveyed two sites, Miyobo located near the center of the country, where only the S molecular form was present, and a coastal site, Ngonamanga, where the M molecular form comprised nearly 90% of the collections. In Miyobo, the susceptible allele (L1014L) frequency was 75%, and L1014F and L1014S allele frequencies were 9% and 16% respectively. In Ngonamanga, the L1014L allele was present in 97% of all *An. gambiae* mosquitoes, with L1014F constituting the remaining 3%. No L1014S alleles were detected among the M molecular forms in this site. Among S forms, the L1014S and L1014F alleles were detected in 40% and 32% of mosquitoes collected, respectively, whereas the L1014L allele was detected in only 32% of S molecular form samples.<sup>13</sup>

In 2007, an extensive pre-intervention survey of vector abundance and insecticide resistance alleles was performed in anticipation of the launch of the EGMCI. Window-mounted exit-traps were used to collect > 4,800 *An. gambiae* sensu lato mosquitoes in > 30 sites throughout continental Equatorial Guinea.<sup>12</sup> The L1014F and L1014S allele frequencies were as high as 59% and 19%, respectively, for S forms, but these allele frequencies were substantially lower for M molecular forms (9.7% for L1014F and 1.8% for L1014S). No *ace-1<sup>R</sup>* alleles were detected among any of the mosquitoes collected in these surveys.<sup>12</sup> The results from this pre-intervention study provided an important baseline and guidance for the sampling strategy for this study.

We report a comparison of levels of *kdr* and *ace-1<sup>R</sup>* resistance alleles before and after the start of a large-scale pyrethroid and carbamate based anti-vector intervention in continental Equatorial Guinea. We analyzed 8,843 mosquitoes collected throughout continental Equatorial Guinea as part of the entomologic monitoring effort of the EGMCI. This analysis was conducted to evaluate whether levels of target site resistance at the *kdr* and *ace-1<sup>R</sup>* loci increased in response to the programmatic use of pyrethroids and carbamates. Studies of this nature provide important insights into the rate of increase in target site resistance under malaria control operations. Furthermore, we discuss the potential epidemiologic impacts of such resistance mechanisms in the current context as efforts to reduce the global burden of malaria intensify.

## METHODS

**Mosquito sampling.** In 2007, before the start of the intervention activities, mosquitoes were collected in window traps affixed to the outside of homes in nine villages (Table 1 and Figure 1). During 2009–2011, mosquitoes were collected by using ultraviolet frequency-modified CDC light traps and human landing collections in residences from 18 sites located throughout continental Equatorial Guinea, including the nine pre-intervention sites (Table 1 and Figure 1).<sup>37</sup>

The average pre- and post-intervention sample sizes were  $n = 91$  and  $n = 401$ , respectively. Data from the village of

TABLE 1

Entomologic monitoring sampling sites, geo-coordinates, and sample size of 2007 and 2009–2011 *Anopheles gambiae* molecular form (M and S) mosquito collections in continental Equatorial Guinea

| Location      |            |              |               | Pre-intervention |       |              | Post-intervention |       |      |     |      |       |                   |
|---------------|------------|--------------|---------------|------------------|-------|--------------|-------------------|-------|------|-----|------|-------|-------------------|
|               |            |              |               | 2007             |       | Total (2007) | 2009              |       | 2010 |     | 2011 |       | Total (2009–2011) |
| Sentinel site | Province   | Latitude (N) | Longitude (E) | M                | S     |              | M                 | S     | M    | S   | M    | S     |                   |
| Aconibe       | Wele Nzas  | 1°16.979'    | 10°54.072'    | –                | 7     | 7            | –                 | 207   | –    | 77  | 3    | 78    | 365               |
| Akurenam      | Centro Sur | 1°2.819'     | 10°37.900'    | 1                | 230   | 231          | 21                | 235   | –    | 109 | 7    | 208   | 580               |
| Anisok        | Wele Nzas  | 1°52.141'    | 10°45.007'    | 63               | 93    | 156          | 1                 | 111   | –    | 84  | 8    | 24    | 228               |
| Cogo          | Litoral    | 1°5.826'     | 9°43.745'     | 11               | 109   | 120          | 123               | 348   | 1    | 6   | 24   | –     | 502               |
| Ebebiyin      | Kie N'tem  | 2°2.703'     | 11°18.757'    | 1                | 365   | 366          | 2                 | 310   | –    | 268 | 6    | 120   | 706               |
| Evinayong     | Centro Sur | 1°20.973'    | 10°34.627'    | –                | –     | –            | 10                | 123   | –    | 14  | –    | 58    | 205               |
| Mongomo       | Wele Nzas  | 1°38.673'    | 11°14.760'    | 1                | 10    | 11           | 3                 | 99    | 1    | 363 | –    | 476   | 942               |
| Ngolo         | Litoral    | 1°51.679'    | 9°47.441'     | 9                | 42    | 51           | 280               | 316   | 136  | 8   | 55   | 44    | 839               |
| Ukomba        | Litoral    | 1°50.604'    | 9°44.730'     | 142              | 40    | 182          | 60                | 69    | 353  | 11  | 50   | 19    | 562               |
| Yengue        | Litoral    | 2°12.630'    | 9°52.489'     | 298              | 209   | 507          | 19                | 202   | 32   | 42  | 35   | 169   | 499               |
| Total         |            |              |               | 526              | 1,105 | 1,631        | 519               | 2,020 | 523  | 982 | 188  | 1,196 | 5,428             |

Ayene was combined with a nearby village, Anisok (10–20 km) to increase the sample size. Sites without pre-intervention data were included in the study to provide a comprehensive picture of the spatial variation of *kdr* and *ace-1<sup>R</sup>* allele frequencies in the region.

**Intervention activities.** Indoor residual spraying included eight and seven spray rounds in Litoral and Kie N'tem Provinces, respectively. Alpha-cypermethrin (a pyrethroid-

class insecticide) was used during rounds 1–4 in Litoral Province and during rounds 1–3 in Kie N'tem Province. Bendiocarb (a carbamate-class insecticide) was applied in round 5 in Litoral Province and rounds 4 and 5 in Kie N'tem Province. Deltamethrin (a pyrethroid-class insecticide) was used during rounds 6–8 in Litoral Province and rounds 6 and 7 in Kie N'tem Province. Deltamethrin-impregnated ITNs were distributed in the two other provinces

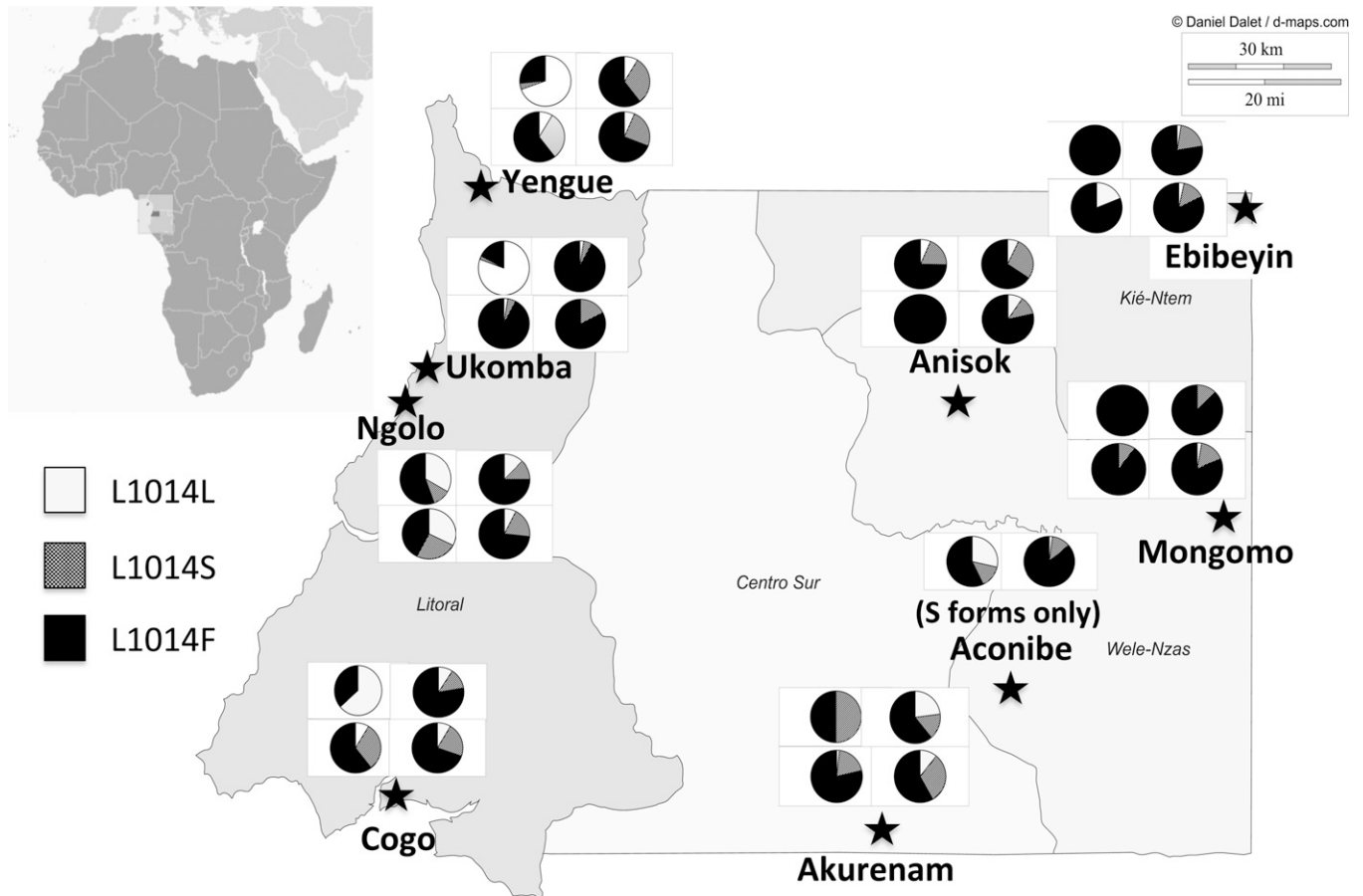


FIGURE 1. Continental Equatorial Guinea showing sampling locations. Stars indicate sites for which pre-intervention and post-intervention sampling was conducted. White squares summarize knockdown resistance (*kdr*) allele frequencies as four pie charts for the M (top) and S (bottom) molecular forms for 2007 (left) and 2009–2011 (right) samples. Circles indicate sampling sites where only post-intervention samples were available.



(Centro Sur and Wele Nzaz). The IRS activities were supplemented with distribution of ITNs in two sites in Litoral province (Mbini, and Cogo), although bed net coverage was low in these sites.

**Molecular genetic analyses.** Anopheline mosquitoes were initially identified to species complex based on morphology and stored in 80% ethanol before transport to the laboratory for molecular analyses. Heads and thoraces were dissected and subjected to DNA extraction by using the Nucleospin<sup>®</sup> 96 Tissue Core extraction kit (Macherey-Nagel and Company, Bethlehem, PA). *Anopheles gambiae* complex mosquitoes were identified to species and molecular form by using polymerase chain reaction (PCR) and restriction fragment length polymorphism assays.<sup>38,39</sup> To determine the presence of *kdr* and *ace-1<sup>R</sup>* alleles, allelic discrimination assays described by Bass and others were performed with an ABI 7500 Fast quantitative PCR instrument (Applied Biosystems, Foster City, CA).<sup>40,41</sup> The Sensimix II<sup>™</sup> (Bioline USA Inc., Taunton, MA) master mixture was used for *kdr* and *ace-1* allele detection assays. TaqMan<sup>®</sup> MGB<sup>™</sup> was obtained from Applied Biosystems. Template DNA volume per reaction was 5  $\mu$ L.

On some occasions, results from this assay were difficult to interpret, and to validate our genotype assignments, PCR amplicons were sequenced from a subset of these samples ( $n = 99$ ). The *kdr* locus was amplified according to conditions described by Pinto and others with slight modifications.<sup>6</sup> Amplicons were purified by using ExoSap-IT (New England BioLabs, Ipswich, MA) and sequenced in both directions by using PCR primers with an ABI3730 instrument (Applied Biosystems). Sequence data were analyzed by using Sequencher<sup>™</sup> version 4.2.2 (Gene Codes Corporation, Ann Arbor, MI). The *kdr* sequences from four strains (GK45, R83, NG05, and GK05) were used as references (GenBank Accession nos. EU078895–U078898).<sup>6</sup>

The *ace-1<sup>R</sup>* allelic discrimination assay described by Bass and others was used to screen for susceptible (*ace-1<sup>S</sup>*) or resistant (*ace-1<sup>R</sup>*) alleles, with minor modifications (5  $\mu$ L of DNA template was used per reaction).<sup>41</sup> Homozygous susceptible and resistant *ace-1* plasmid controls were used as controls. Heterozygous controls were created by mixing equimolar amounts of homozygous resistant and susceptible plasmid DNA.

**Data analyses.** Field-derived geo-coordinates were plotted to create a collection site map by using d-maps.com.<sup>42</sup> Allele and genotype frequencies for the *kdr* locus were calculated by using GENEPOP.<sup>43</sup> Chi-square analyses were performed to determine differences in genotype frequencies between years. Hardy-Weinberg (HW) equilibrium analyses were performed on populations for which > 10 mosquitoes were sampled by using Arlequin (10,000 permutations).<sup>44</sup> A Z-test of proportions was performed to determine relative changes in allele frequency proportions pre-intervention and post-intervention by using the In-Silico<sup>™</sup> online statistical calculator.<sup>45</sup>

To explore a possible explanation for HW disequilibria in the *kdr* locus in some populations, microsatellite data for 16 loci for the M form (Ukomba,  $n = 93$ ) and the S form (Yengue,  $n = 62$ ) collected in 2007 were used to perform a Bayesian clustering analysis implemented in STRUCTURE version 2.3.3.<sup>46,47</sup> Each STRUCTURE analysis was run for 500,000 generations, with a burn-in period of 100,000 generations by using correlated allele frequencies with no *a priori*

population information. The number of potential distinct genetic clusters (K) examined ranged from 1 to 5 and 5 replicates were run for each K value. STRUCTURE Harvester was used to examine the STRUCTURE output.<sup>48</sup>

## RESULTS

A total of 7,059 *An. gambiae* mosquitoes were identified from our collections and analyzed for *kdr* and *ace-1<sup>R</sup>* alleles. The 2007 window trap collections provided 1,631 *An. gambiae*, and the 2009–2011 human landing catches and light trap collections provided 5,428 *An. gambiae*. In none of the *An. gambiae* mosquitoes collected before or following the intervention were any *ace-1<sup>R</sup>* alleles detected. Quantitative PCR calls were determined to be in agreement with sequence data in 96 (97.0%) of 99 samples.

The *kdr* allele frequencies for populations for which pre-intervention and post-intervention data were available are shown in Table 2 and Supplemental Table 1. The *kdr* alleles were already at considerable frequencies in *An. gambiae* populations in continental Equatorial Guinea before the intervention. The L1014S allele was detected in all but a single M form population (Cogo) (Table 2) and all S form populations (Table 2). Frequencies of this allele were much lower in the M form than in the S form, ranging from 0% to 19.0% in the M form and from 10.0% to 42.9% in the S form. Although L1014F frequencies were generally high, S and M form populations showed remarkable within-form variation in the frequency of this allele. This variation ranged in frequency from 17.6% to 74.6% in the M form, and from 27.1% to as high as 90.0% in S form. Consequently, the frequency of L1014L, also showed considerable variation before the intervention, as in 2007, ranged from 6.3% to 80.3% and from 0% to 61.5% in the M and S forms, respectively (Table 2).

The L1014S allele showed a higher frequency in the S form in all five locations for which data from both forms were available. However, this difference was significant only for Ngolo, Ukomba, and Yengue ( $P < 0.011$ , by Z-test). Such a pattern was not observed for the L1014F allele, for which only two of the five locations showed a significantly higher frequency for the S form (Ukomba and Yengue;  $P < 0.0001$ , by Z-test). In the other three populations, M form samples had a higher frequency of L1014F, although these differences were not significant.

After several years of pyrethroid application in the region, the frequency of L1014F increased in all five M form populations for which pre-intervention and post-intervention data were available (73.3% and 100%, respectively, in 2009–2011) (Table 2 and Supplemental Figure 3). This increase was significant in three of the five populations. The two populations (Anisok and Ngolo) for which the increase was not significant had low sample sizes for one of the collection years. In only one M form population (Yengue), a barely significant increase in L1014S frequency was observed.

An increase in combined *kdr* allele frequencies and a corresponding reduction in the susceptible allele L1014L was also observed in all S form populations in which L1014L was still present at a frequency > 10% in 2007 (Aconibe, Akurenam, Cogo, and Ukomba;  $P < 0.001$ , by Z-test) (Table 2 and Supplemental Figure 4). In the S form in Cogo, the most extreme example, the frequency of L1014F

TABLE 2

Frequencies of *kdr* alleles in *Anopheles gambiae* M form (A) and S form (B) populations in continental Equatorial Guinea for which pre-intervention (2007) and post-intervention (2009–2011) data were available\*

| Site (A) | Intervention | 2007 | 2009–2011 | 2007   | 2009–2011 | Trend    | 2007   | 2009–2011 | Trend    | 2007   | 2009–2011 | Trend    |
|----------|--------------|------|-----------|--------|-----------|----------|--------|-----------|----------|--------|-----------|----------|
|          |              | 2N   | 2N        | L1014L | L1014L    | L1014L   | L1014S | L1014S    | L1014S   | L1014F | L1014F    | L1014F   |
| Anisok   | ITN          | 126  | 18        | 6.3%   | 11.1%     | –        | 19.0%  | 5.6%      | –        | 74.6%  | 83.3%     | –        |
| Cogo     | IRS          | 22   | 296       | 63.6%  | 9.1%      | Decrease | 0.0%   | 13.2%     | –        | 36.4%  | 77.7%     | Increase |
| Ngolo    | IRS          | 18   | 942       | 33.3%  | 12.4%     | Decrease | 11.1%  | 12.6%     | –        | 55.6%  | 74.9%     | –        |
| Ukomba   | IRS          | 284  | 926       | 80.3%  | 2.5%      | Decrease | 2.1%   | 5.2%      | Increase | 17.6%  | 92.3%     | Increase |
| Yengue   | IRS          | 596  | 172       | 69.5%  | 19.8%     | Decrease | 4.0%   | 7.0%      | –        | 26.5%  | 73.3%     | Increase |
| Average  |              |      |           | 50.6%  | 11.0%     |          | 7.3%   | 8.7%      |          | 42.1%  | 80.3%     |          |

| Site (B) | Intervention | 2007 | 2009–2011 | 2007   | 2009–2011 | Trend    | 2007   | 2009–2011 | Trend    | 2007   | 2009–2011 | Trend    |
|----------|--------------|------|-----------|--------|-----------|----------|--------|-----------|----------|--------|-----------|----------|
|          |              | 2N   | 2N        | L1014L | L1014L    | L1014L   | L1014S | L1014S    | L1014S   | L1014F | L1014F    | L1014F   |
| Aconibe  | ITN          | 14   | 724       | 28.6%  | 1.9%      | Decrease | 14.3%  | 11.7%     | –        | 57.1%  | 86.3%     | Increase |
| Akurenam | ITN          | 460  | 1,104     | 23.0%  | 10.4%     | Decrease | 15.9%  | 30.3%     | Increase | 61.1%  | 59.2%     | –        |
| Anisok   | ITN          | 176  | 438       | 6.8%   | 8.9%      | –        | 27.8%  | 12.1%     | Decrease | 65.3%  | 79.0%     | Increase |
| Mongomo  | ITN          | 20   | 1,876     | 0.0%   | 2.8%      | –        | 10.0%  | 15.9%     | –        | 90.0%  | 81.3%     | –        |
| Cogo     | IRS          | 218  | 1,068     | 61.5%  | 8.0%      | Decrease | 11.5%  | 20.0%     | Increase | 27.1%  | 72.0%     | Increase |
| Ebebiyin | IRS          | 730  | 1,396     | 2.5%   | 3.5%      | –        | 19.9%  | 14.1%     | Decrease | 77.7%  | 82.4%     | Increase |
| Ngolo    | IRS          | 84   | 736       | 2.4%   | 3.1%      | –        | 42.9%  | 15.1%     | Decrease | 54.8%  | 81.8%     | Increase |
| Ukomba   | IRS          | 80   | 218       | 32.5%  | 0.0%      | Decrease | 25.0%  | 25.7%     | –        | 42.5%  | 74.3%     | Increase |
| Yengue   | IRS          | 418  | 826       | 8.6%   | 7.0%      | –        | 30.9%  | 24.5%     | Decrease | 60.5%  | 68.5%     | Increase |
| Average  |              |      |           | 18.4%  | 5.1%      |          | 22.0%  | 18.8%     |          | 59.6%  | 76.1%     |          |

\**kdr* = knockdown resistance; ITN = insecticide-treated net; IRS = indoor residual spraying. Significant differences between years for each of the three alleles (L1014L, L1014S, and L1014F) are indicated by increase or decrease in the Trend column. *P* values are shown in Supplemental Table 1.

and L1014S increased from 27.1% to 72.0%, and from 11.5% to 20.0%, respectively. However, if L1014L was already at low frequency in pre-intervention samples, no significant decreases in L1014L were observed. Populations that were subjected to IRS and ITNs showed increases in L1014S and/or L1014F, and no obvious difference between the two control methods on the increase of *kdr* alleles was evident in our data.

To determine whether changes in *kdr* allele frequencies are best explained by the action of genetic drift rather than selection, we examined changes in allele frequencies for 16 microsatellite loci in the Mongomo, Yengue, and Ukomba populations during 2007–2010 (Supplemental Table 2).<sup>46</sup> For

this dataset, sample sizes ranged from 36 to 93, with averages of 57, 50, and 82, respectively. The average change in frequency for all microsatellite alleles was 0.040, 0.055, and 0.038, respectively. In contrast, the average change in the frequency of L1014S between 2007 and 2009–2011 was 0.282. We excluded populations that had a sample size < 50 for one of the time points in calculating the average increase in L1014F to avoid large sampling effects. In addition, in eight of the nine populations, the frequency of L1014F increased. It therefore is unlikely that the observed increase in L1014F is explained by genetic drift.

The *kdr* allele frequency data for populations for which only post-intervention data were available are shown in

TABLE 3

Frequencies of *kdr* alleles in *Anopheles gambiae* M molecular form (A) and S molecular form (B) populations in continental Equatorial Guinea, 2009–2011, following several years of vector control\*

| Site (A)   | Intervention | 2N  | L1014L | 95% CI | L1014S | 95% CI | L1014F | 95% CI |
|------------|--------------|-----|--------|--------|--------|--------|--------|--------|
| Akurenam   | ITN          | 56  | 1.8%   | 3.5    | 19.6%  | 10.4   | 78.6%  | 10.7   |
| Bicurga    | ITN          | 40  | 0.0%   | –      | 20.0%  | 12.4   | 80.0%  | 12.4   |
| Evinayong  | ITN          | 20  | 0.0%   | –      | 20.0%  | 17.5   | 80.0%  | 17.5   |
| Mongomo    | ITN          | 8   | 0.0%   | –      | 12.5%  | 22.9   | 87.5%  | 22.9   |
| Niefang    | ITN          | 16  | 18.8%  | 19.1   | 25.0%  | 21.1   | 56.3%  | 24.3   |
| Ayamiken   | IRS          | 54  | 0.0%   | –      | 18.5%  | 10.4   | 81.5%  | 10.4   |
| Ebebiyin   | IRS          | 16  | 18.8%  | 19.1   | 0.0%   | –      | 81.3%  | 19.1   |
| Etofilii   | IRS          | 40  | 0.0%   | –      | 22.5%  | 12.9   | 77.5%  | 12.9   |
| Mbini      | IRS          | 196 | 8.2%   | 3.8    | 6.1%   | 3.4    | 85.7%  | 4.9    |
| Site (B)   | Intervention | 2N  | L1014L | 95% CI | L1014S | 95% CI | L1014F | 95% CI |
| Bicurga    | ITN          | 332 | 0.3%   | 0.6    | 28.6%  | 4.9    | 71.1%  | 4.9    |
| Evinayong  | ITN          | 390 | 4.9%   | 2.1    | 26.4%  | 4.4    | 68.7%  | 4.6    |
| Niefang    | ITN          | 634 | 9.3%   | 2.3    | 32.6%  | 3.7    | 58.0%  | 3.8    |
| Nsork      | ITN          | 244 | 22.1%  | 5.2    | 11.1%  | 3.9    | 66.8%  | 5.9    |
| Ayamiken   | IRS          | 476 | 0.8%   | 0.8    | 39.1%  | 4.4    | 60.1%  | 4.4    |
| Etofilii   | IRS          | 62  | 0.0%   | –      | 32.3%  | 11.6   | 67.7%  | 11.6   |
| Mbini      | IRS          | 484 | 4.1%   | 1.8    | 13.8%  | 3.1    | 82.0%  | 3.4    |
| Micomeng   | IRS          | 148 | 4.7%   | 3.4    | 37.8%  | 7.8    | 57.4%  | 7.8    |
| Nsok Nsomo | IRS          | 484 | 1.7%   | 1.2    | 15.5%  | 3.2    | 82.9%  | 3.4    |
| Average    |              |     | 5.3%   |        | 26.4%  |        | 68.3%  |        |

\**kdr* = knockdown resistance; CI = confidence interval; ITN = insecticide-treated net; IRS = indoor residual spraying.

Table 3. In all thirteen M form populations sampled during 2009–2011, the combined *kdr* frequencies ranged between 81.2% and 100%, averaging 93.0% (Tables 2 and 3 and Supplemental Figure 3). This high frequency was mostly caused by L1014F, which averaged 81.8%, and ranged from 56.3% and 100% in 2009–2011. In the eighteen S form populations examined in 2009–2011, combined *kdr* frequencies ranged between 77.9% and 100%, averaging 94.6% (Tables 2 and 3 and Supplemental Figure 4). Similar to the M form, although to a lesser extent, this finding was also caused by high frequencies of L1014F, which ranged from 57.4% to 86.1%, averaging 72.2%.

In five S form populations (Anisok, Mongomo, Ebebiyin, Ngolo, and Yengue) and one M form population (Anisok), the L1014L allele frequency was low (< 8.6%) before the start of the intervention. In these six populations, L1014S and L1014F were present, but L1014F showed a much higher frequency (68.5–83.3%). Interestingly, in four of these six populations the L1014S frequency decreased significantly ( $P < 0.0006$ , by *Z*-test) after the start of the intervention, and the L1014F frequency increased significantly ( $P < 0.0001$ , by *Z*-test). In the two exceptions, Anisok (M form) and Mongomo (S form), few samples were available for 2007 or 2009–2011, and no significant differences between time points were observed for either *kdr* allele. Only two other populations showed significant differences in L1014S frequency between years (Ukomba, M form and Akurenam, S form), and in both these populations the frequency of this allele increased. However, frequencies of L1014L were rela-

tively high in these two populations in 2007 (80.3% and 23.0%, respectively).

The *kdr* genotype frequencies for M form populations are shown in Table 4. Almost all M form samples did not show HW equilibrium. The 2007 samples showed an excess of L1014L and L1014F homozygotes. In 2009, the pattern was somewhat different. For example, Ukomba in 2009 had a deficit of L1014S homozygotes, whereas Ngolo had an excess of L1014S and L1014F homozygotes. However, in 2010, Ngolo had a slight deficit of L1014F homozygotes. In 2011, Ngolo again did not show HW equilibrium (although not after Bonferroni correction), with an excess of L1014L and L1014F homozygotes. Thus, although most samples did not show HW disequilibrium, there is no consistent pattern regarding what could be considered missing genotypes.

The *kdr* genotype frequencies for S form populations are shown in Table 5. Again, almost all populations were not in HW equilibrium. Frequently, this finding was caused by a deficiency of L1014S homozygotes (e.g., Akurenam in 2009 or Cogo in 2009), but in other cases an excess of L1014S homozygotes was observed (e.g., Ngolo in 2009). Again, no consistent pattern regarding missing genotypes emerged.

A possible explanation for the observed HW disequilibria could be population subdivision within M and S form populations. We therefore conducted a Bayesian clustering analyses by using microsatellite data from *An. gambiae* collected in Ukomba (M form) and Yengue (S form) in 2007.<sup>46</sup> No subdivision was detected between these two populations (Supplemental Figures 1 and 2), although both exhibited

TABLE 4

Genotype frequencies and *P* values for chi-square tests for deviation from Hardy-Weinberg equilibrium for *Anopheles gambiae* M molecular forms collected in continental Equatorial Guinea, in 2007 and 2009–2011\*

| 2007 Pre-intervention  |     | Genotype |     |     |     |     |     |       |          |           |                |
|------------------------|-----|----------|-----|-----|-----|-----|-----|-------|----------|-----------|----------------|
| Site                   | L/L | L/S      | S/S | L/F | S/F | F/F | N   | $H_O$ | $H_{ES}$ | $F_{ISS}$ | <i>P</i>       |
| Anisok                 | 4   | 0        | 0   | 0   | 24  | 35  | 63  | 0.38  | 0.41     | 0.06      | < <b>0.001</b> |
| Cogo                   | 7   | 0        | 0   | 0   | 0   | 4   | 11  | 0.00  | 0.48     | 1.00      | <b>0.001</b>   |
| Ukomba                 | 114 | 0        | 0   | 0   | 6   | 22  | 142 | 0.04  | 0.31     | 0.86      | < <b>0.001</b> |
| Yengue                 | 201 | 1        | 3   | 11  | 17  | 65  | 298 | 0.10  | 0.44     | 0.78      | < <b>0.001</b> |
| Total                  | 326 | 1        | 3   | 11  | 47  | 126 | 514 |       |          |           |                |
| 2009 Post-intervention |     | Genotype |     |     |     |     |     |       |          |           |                |
| Site                   | L/L | L/S      | S/S | L/F | S/F | F/F | N   | $H_O$ | $H_{ES}$ | $F_{ISS}$ | <i>P</i>       |
| Akurenam               | 0   | 0        | 0   | 0   | 6   | 15  | 21  | 0.28  | 0.25     | -0.14     | 1.000          |
| Cogo                   | 5   | 0        | 2   | 5   | 31  | 80  | 123 | 0.29  | 0.34     | 0.15      | < <b>0.001</b> |
| Ngolo                  | 0   | 3        | 17  | 7   | 40  | 213 | 280 | 0.18  | 0.27     | 0.33      | < <b>0.001</b> |
| Ukomba                 | 0   | 0        | 0   | 0   | 43  | 17  | 60  | 0.72  | 0.46     | -0.55     | < <b>0.001</b> |
| Yengue                 | 1   | 4        | 0   | 0   | 5   | 9   | 19  | 0.47  | 0.57     | 0.17      | <b>0.005</b>   |
| Total                  | 6   | 7        | 19  | 12  | 125 | 334 | 503 |       |          |           |                |
| 2010 Post-intervention |     | Genotype |     |     |     |     |     |       |          |           |                |
| Site                   | L/L | L/S      | S/S | L/F | S/F | F/F | N   | $H_O$ | $H_{ES}$ | $F_{ISS}$ | <i>P</i>       |
| Ngolo                  | 2   | 2        | 17  | 81  | 6   | 28  | 136 | 0.65  | 0.60     | -0.09     | < <b>0.001</b> |
| Ukomba                 | 1   | 0        | 0   | 15  | 5   | 332 | 353 | 0.06  | 0.06     | 0.07      | 0.286          |
| Yengue                 | 0   | 0        | 0   | 0   | 3   | 29  | 32  | 0.09  | 0.09     | -0.03     | 1.000          |
| Total                  | 3   | 2        | 17  | 96  | 14  | 389 | 521 |       |          |           |                |
| 2011 Post-intervention |     | Genotype |     |     |     |     |     |       |          |           |                |
| Site                   | L/L | L/S      | S/S | L/F | S/F | F/F | N   | $H_O$ | $H_{ES}$ | $F_{ISS}$ | <i>P</i>       |
| Cogo                   | 6   | 0        | 0   | 0   | 4   | 14  | 24  | 0.17  | 0.50     | 0.67      | < <b>0.001</b> |
| Ngolo                  | 10  | 0        | 0   | 0   | 0   | 45  | 55  | 0.00  | 0.30     | 1.00      | < <b>0.001</b> |
| Ukomba                 | 3   | 0        | 0   | 0   | 1   | 46  | 50  | 0.02  | 0.13     | 0.85      | < <b>0.001</b> |
| Yengue                 | 8   | 0        | 0   | 12  | 0   | 15  | 35  | 0.34  | 0.49     | 0.30      | 0.845          |
| Total                  | 27  | 0        | 0   | 12  | 5   | 120 | 164 |       |          |           |                |

\*Significant *P* values are indicated in bold.

TABLE 5

Genotype frequencies and *P* values for chi-square tests for deviation from Hardy-Weinberg equilibrium for *Anopheles gambiae* S molecular forms collected in continental Equatorial Guinea, 2007 and 2009–2011\*

| 2007 Pre-intervention  |     | Genotype |     |     |     |       |       |       |          |           |                |
|------------------------|-----|----------|-----|-----|-----|-------|-------|-------|----------|-----------|----------------|
| Site                   | L/L | L/S      | S/S | L/F | S/F | F/F   | N     | $H_O$ | $H_{ES}$ | $F_{ISS}$ | <i>P</i>       |
| Akurenam               | 53  | 0        | 1   | 0   | 71  | 105   | 230   | 0.31  | 0.55     | 0.44      | < <b>0.001</b> |
| Anisok                 | 6   | 0        | 5   | 0   | 39  | 38    | 88    | 0.45  | 0.45     | -0.01     | < <b>0.001</b> |
| Cogo                   | 67  | 0        | 5   | 0   | 15  | 22    | 109   | 0.12  | 0.51     | 0.77      | < <b>0.001</b> |
| Ebebiyin               | 9   | 0        | 9   | 0   | 127 | 220   | 365   | 0.35  | 0.35     | -0.02     | < <b>0.001</b> |
| Mongomo                | 0   | 0        | 0   | 0   | 2   | 8     | 10    | 0.29  | 0.26     | -0.09     | 1.000          |
| Ngolo                  | 1   | 0        | 7   | 0   | 22  | 12    | 42    | 0.52  | 0.52     | 0.00      | <b>0.019</b>   |
| Ukomba                 | 13  | 0        | 5   | 0   | 10  | 12    | 40    | 0.21  | 0.66     | 0.68      | < <b>0.001</b> |
| Yengue                 | 14  | 1        | 18  | 7   | 92  | 77    | 209   | 0.48  | 0.53     | 0.10      | < <b>0.001</b> |
| Total                  | 163 | 1        | 50  | 7   | 378 | 494   | 1,093 |       |          |           |                |
| 2009 Post-intervention |     | Genotype |     |     |     |       |       |       |          |           |                |
| Site                   | L/L | L/S      | S/S | L/F | S/F | F/F   | N     | $H_O$ | $H_{ES}$ | $F_{ISS}$ | <i>P</i>       |
| Aconibe                | 0   | 0        | 1   | 2   | 46  | 158   | 207   | 0.231 | 0.217    | -0.08     | 0.470          |
| Akurenam               | 2   | 0        | 3   | 2   | 141 | 87    | 235   | 0.608 | 0.448    | -0.36     | < <b>0.001</b> |
| Anisok                 | 0   | 0        | 1   | 0   | 18  | 92    | 111   | 0.16  | 0.165    | 0.02      | 1.000          |
| Cogo                   | 10  | 0        | 5   | 9   | 132 | 192   | 348   | 0.405 | 0.388    | -0.04     | < <b>0.001</b> |
| Ebebiyin               | 5   | 1        | 5   | 1   | 70  | 228   | 310   | 0.232 | 0.261    | 0.11      | < <b>0.001</b> |
| Mongomo                | 1   | 1        | 2   | 1   | 53  | 41    | 99    | 0.555 | 0.444    | -0.25     | < <b>0.001</b> |
| Ngolo                  | 1   | 3        | 18  | 9   | 49  | 236   | 316   | 0.193 | 0.277    | 0.30      | < <b>0.001</b> |
| Ukomba                 | 0   | 0        | 0   | 0   | 47  | 22    | 69    | 0.68  | 0.45     | -0.51     | < <b>0.001</b> |
| Yengue                 | 1   | 9        | 1   | 0   | 122 | 69    | 202   | 0.648 | 0.477    | -0.36     | < <b>0.001</b> |
| Total                  | 20  | 14       | 36  | 24  | 678 | 1,125 | 1,897 |       |          |           |                |
| 2010 Post-intervention |     | Genotype |     |     |     |       |       |       |          |           |                |
| Site                   | L/L | L/S      | S/S | L/F | S/F | F/F   | N     | $H_O$ | $H_{ES}$ | $F_{ISS}$ | <i>P</i>       |
| Aconibe                | 0   | 1        | 0   | 0   | 5   | 71    | 77    | 0.078 | 0.087    | 0.11      | 0.088          |
| Akurenam               | 5   | 19       | 4   | 35  | 41  | 5     | 109   | 0.87  | 0.663    | -0.31     | < <b>0.001</b> |
| Anisok                 | 15  | 0        | 3   | 4   | 20  | 42    | 84    | 0.285 | 0.524    | 0.46      | < <b>0.001</b> |
| Ebebiyin               | 3   | 1        | 4   | 1   | 82  | 177   | 268   | 0.313 | 0.307    | -0.02     | < <b>0.001</b> |
| Mongomo                | 3   | 0        | 1   | 3   | 50  | 306   | 363   | 0.146 | 0.155    | 0.06      | < <b>0.001</b> |
| Ukomba                 | 0   | 0        | 0   | 0   | 0   | 11    | 11    | 0     | 0        | -         | 1.000          |
| Yengue                 | 1   | 0        | 0   | 0   | 5   | 36    | 42    | 0.119 | 0.157    | 0.25      | <b>0.25</b>    |
| Total                  | 27  | 21       | 12  | 43  | 203 | 648   | 954   |       |          |           |                |
| 2011 Post-intervention |     | Genotype |     |     |     |       |       |       |          |           |                |
| Site                   | L/L | L/S      | S/S | L/F | S/F | F/F   | N     | $H_O$ | $H_{ES}$ | $F_{ISS}$ | <i>P</i>       |
| Aconibe                | 3   | 3        | 4   | 2   | 20  | 46    | 78    | 0.32  | 0.42     | 0.25      | < <b>0.001</b> |
| Akurenam               | 8   | 9        | 14  | 20  | 83  | 74    | 208   | 0.538 | 0.542    |           | <b>0.002</b>   |
| Anisok                 | 1   | 0        | 0   | 3   | 7   | 13    | 24    | 0.417 | 0.414    | -0.01     | 0.988          |
| Cogo                   | 21  | 6        | 8   | 6   | 48  | 91    | 180   | 0.333 | 0.511    | 0.35      | < <b>0.001</b> |
| Ebebiyin               | 13  | 3        | 2   | 0   | 18  | 84    | 120   | 0.175 | 0.375    | 0.53      | < <b>0.001</b> |
| Mongomo                | 6   | 3        | 15  | 24  | 155 | 273   | 476   | 0.382 | 0.379    | 0.00      | < <b>0.001</b> |
| Ngolo                  | 2   | 4        | 2   | 0   | 6   | 30    | 44    | 0.227 | 0.408    | 0.45      | < <b>0.001</b> |
| Ukomba                 | 0   | 0        | 2   | 0   | 5   | 12    | 19    | 0.263 | 0.371    | 0.30      | 0.235          |
| Yengue                 | 5   | 3        | 6   | 32  | 49  | 74    | 169   | 0.50  | 0.488    | -0.02     | 0.079          |
| Total                  | 59  | 31       | 53  | 87  | 391 | 697   | 1,318 |       |          |           |                |

\*Significant *P* values are indicated in **bold**.

significant deviation from HW equilibrium at the *kdr* locus in 2007.

## DISCUSSION

This study examined target site resistance against two insecticide classes in *An. gambiae* within an operational context in which IRS and ITN interventions were applied on a nationwide scale. We identified a dramatic increase in the L1014F allele in nearly all sites for which pre-intervention and post-intervention data were available, but no increase in the L1014S allele in the same sites. In sites for which only post-intervention data were available, the frequency of L1014F was high in 2009–2011 for M and S forms. These data are consistent with strong selection imposed on *An. gambiae*

populations by use of pyrethroid insecticides in anti-malarial interventions also reported in other studies.<sup>16,18,22</sup>

Interestingly, if the combined pre-intervention frequency of L1014S and L1014F was high, L1014S frequencies decreased after the start of the intervention. These observations strongly suggest that L1014S can increase at the expense of the susceptible L1014L allele, but that it tends to decrease when competing primarily with L1014F. That being said, the S form population in Akurenam does not fully match the pattern described above because the frequency of L1014F remained the same while that of L1014S increased at the expense of L1014L. However, our results suggest that although L1014F and L1014S have a higher fitness than the susceptible L1014L in the presence of pyrethroid insecticides, the fitness of L1014F is substantially higher than that of L1014S, at least



in the presence of alpha-cypermethrin and deltamethrin. From this result it follows that L1014F should have a larger impact on the efficacy of pyrethroid-based vector control. This idea is consistent with circumstantial evidence suggesting that L1014F provides more protection against pyrethroids.<sup>2,6,49,50</sup> Furthermore, we should expect to see a further decrease in L1014S frequency in *An. gambiae* populations as L1014F gets closer towards fixation. However, we stress that additional data are needed to establish these inferences beyond doubt.

Although no programmatic use of pyrethroids was implemented on continental Equatorial Guinea before control activities, *kdr* was present at considerable frequencies in *An. gambiae* populations. Ridl and others reported the presence of L1014F and L1014S alleles in *An. gambiae* populations across continental Equatorial Guinea sampled in December 2006–July 2007.<sup>12</sup> Although sample sizes were low for many of the populations included in that study, L1014F clearly had a much higher frequency than L1014S for both forms. This finding contrasts with results for two *An. gambiae* S form populations collected in 2004–2005, in which L1014S had a higher frequency than L1014F.<sup>13</sup> However, in both these studies L1014F frequencies were much lower in the M form than we observed, ranging only between 0% and 3% and 7.5% and 18.2%, underscoring the impact of pyrethroid use on *kdr* frequencies. As was observed in our study, Ridl and others also detected considerable heterogeneity of *kdr* frequencies across continental Equatorial Guinea.<sup>12</sup>

These high pre-intervention levels of *kdr* alleles raise the question why these resistance alleles were present at such high frequencies in continental Equatorial Guinea. Unlike neighboring Cameroon, where agricultural uses of insecticides constitute an important proportion of pyrethroid use, Equatorial Guinea relies mostly on subsistence agriculture and has limited agro-industrial capacity.<sup>51</sup> However, information on insecticide use for agricultural purposes is not available for Equatorial Guinea.<sup>52</sup> Previous anti-vector interventions have been limited to intermittent bed net distributions with little, if any, follow-up monitoring for insecticide resistance conducted by the agencies responsible for their deployment.

The *kdr* alleles were most likely introduced into Equatorial Guinea through neighboring countries (Cameroon or Gabon). In four villages in southern Cameroon, L1014F and L1014S were present in S form populations in 2007, and L1014F had a considerably higher frequency than L1014S.<sup>30</sup> These villages were sampled across a diverse landscape of urban and agro-industrial settings in which pyrethroids and other insecticides are commonly used for agricultural, household nuisance reduction and public health uses, and L1014F increased substantially during 2003–2007. However, L1014S was not found in four M form populations collected during 2003–2007, and only since 2007 was L1014F present in three of these M form populations.<sup>30</sup>

In Gabon, which borders Equatorial Guinea to the south, Pinto and others observed a much higher frequency of L1014S (63.0%) than L1014F (37.0%) in an S form population only approximately 100 km from the southern border of Equatorial Guinea.<sup>8</sup> Therefore, Gabon provides another possible entry route for L1014F and/or L1014S. However, the pre-intervention frequencies of *kdr* alleles in continental

Equatorial Guinea are much higher than can be explained by mosquito migration and several years of genetic drift. That is, a neutral allele that is introduced into a large population is unlikely to reach such a high frequency in a few years through genetic drift. Therefore, it is likely that *kdr* alleles were already under considerable selection pressure from pyrethroid use, which is readily available for pest control. Nonetheless, use of pyrethroids was greatly increased by initiation of the EGMC, and L1014F frequencies in S and M form populations continued to increase and have reached high levels in a few years after the start of the campaign.

The main issue at hand is the effect of *kdr* alleles on the efficacy of pyrethroid-based vector control. The selection pressure by pyrethroid use on L1014F in particular is obviously strong, resulting in frequencies close to fixation after only a few years of interventions. A correlation between the *kdr* genotype and resistance phenotype has also been established.<sup>53</sup> However, this correlation does not necessarily mean that the presence of *kdr* prevents pyrethroid-based vector control from having a large impact. For example, in Burundi, a vector control program based on pyrethroids greatly reduced malaria transmission despite the presence of the L1014S allele at a high frequency.<sup>54,55</sup> In Côte d'Ivoire, where a L1014F allele frequency > 80% was observed in *An. gambiae*, pyrethroid-treated bed nets still provided a protective effect.<sup>1</sup> In a recent experimental hut study conducted in Benin, pyrethroid-treated ITNs containing holes were shown to provide a significant reduction in the blood-feeding rate (42%) of highly *kdr*-resistant (84%) *An. gambiae*, suggesting that even when ITNs are compromised because of wear and tear, pyrethroids still offer some measure of protection to persons sleeping under them.<sup>56</sup>

Recently, a modeling study by Kiszewski and others quantified the expected effect of *kdr* on vector control efficacy (Kiszewski AE and others, unpublished data). That study showed that the presence of *kdr* during an IRS campaign results in a substantially higher entomologic inoculation rate compared with when the campaign was absent. However, the study also showed that IRS in the presence of *kdr* still is expected to provide a large reduction in the entomologic inoculation rate. Furthermore, Athrey and others examined the effect of IRS and ITN on the effective population sizes of three *An. gambiae* populations in Equatorial Guinea, and found that these were reduced approximately 57%, 82%, and 85% around the start of the control, despite the presence of *kdr* at high frequencies.<sup>46</sup> Taken together, these findings indicate that anti-vector interventions based on pyrethroids do not cease to be effective solely because of the presence of *kdr* alleles, even though *kdr* is expected to have a negative impact on efficacy of control. That being said, a study with *Culex pipiens* provided a warning by showing a synergistic effect between *kdr* and metabolic resistance, resulting in high resistance against pyrethroids.<sup>57</sup>

The *ace-1<sup>R</sup>* gene, which provides resistance against carbamate insecticides, has been reported in *An. gambiae* populations in west Africa.<sup>28,34,36</sup> For example, in Burkina Faso, *ace-1<sup>R</sup>* alleles have reached frequencies in excess of 30% in S form populations and 3% in M form populations.<sup>34</sup> In Côte d'Ivoire, frequencies of this allele have reached 30.9% and 35.2% among M and S forms, respectively.<sup>36</sup> Apparently this finding is caused mostly by agricultural use of carbamate- and organophosphate-class insecticides. Because

*kdr* alleles were found throughout much of the range of *An. gambiae* shortly after their discovery, the presence of *ace-1<sup>R</sup>* was monitored in Equatorial Guinea. Fortunately, no *ace-1<sup>R</sup>* alleles were found in any of the pre-intervention and post-intervention samples analyzed. This finding is consistent with the absence of *ace-1<sup>R</sup>* in samples from Equatorial Guinea analyzed by Ridl and others,<sup>12</sup> and the absence of the allele on Bioko Island, where carbamates have been used as part of an ongoing IRS campaign since 2005.<sup>58</sup>

The level of resistance provided by *ace-1<sup>R</sup>* to various carbamate insecticides is high and therefore has the potential to greatly undermine IRS efficacy.<sup>59</sup> In addition, a duplicated allele, *ace-1<sup>D</sup>* was recently identified and found to reach frequencies  $\leq 65\%$  in Côte d'Ivoire and Burkina Faso, and is likely present in Benin.<sup>29</sup> The implication of the *ace-1<sup>R</sup>* allele duplication is that it produces a permanent heterozygote, which lessens the fitness deficit attributed to *ace-1<sup>R</sup>*.<sup>29</sup> Given the current absence of *ace-1<sup>R</sup>*, carbamate insecticides remain a suitable choice as part of IRS operations in Equatorial Guinea. However, given the potential of this form of target site resistance to spread and undermine carbamate-based vector control, it is advisable that *An. gambiae* populations in Equatorial Guinea continue to be monitored for the presence these alleles.

No population structure is evident within the molecular forms of *An. gambiae* on the geographic scale of this study.<sup>60,61</sup> Based on available microsatellite datasets, we found no significant differentiation between the S form in Yengue and Mongomo, two populations 150 km apart.<sup>46</sup> This finding could raise the question whether our populations represent independent samples. Considerable gene flow may prevent neutral microsatellite loci from diverging, but given that individual mosquito migration is typically estimated to be at most 2 km, it is difficult to conceive gene flow being high enough that *kdr* alleles selected for in one population increase correspondingly in a population 150 km apart in the short time span covered in this study.

In most of our collections, populations showed HW disequilibrium for the *kdr* locus. Several potential explanations could be offered for this observation. The first is possible technical error, which would result in some genotypes consistently not getting scored. Our sequencing validation indicates that this is not a likely explanation because even in genotype calls that we considered difficult, error rates were low. In addition, no clear pattern of specific missing genotypes emerged from our data. Another possible explanation could be previously undetected population subdivision within the M and S forms in Equatorial Guinea, an unlikely explanation that is ruled out by our Bayesian clustering analyses (Supplemental Figures 1 and 2). Finally, selection for specific genotypes in combination with variation in the frequency of alleles throughout the year might explain the observed deviations. It has been reported before that *kdr* allele frequencies can vary seasonally, and in our data, samples were pooled for an entire year.<sup>19,62,63</sup> We also have shown that selection has acted strongly on this locus after the application of pyrethroid insecticides.

In conclusion, our analyses of *An. gambiae* mosquitoes collected during the vector monitoring component of the EGMCI, indicate that L1014F was already present at substantial frequencies before the start of a large pyrethroid-based IRS and ITN distribution campaign. However, L1014F

increased dramatically within the first few years of vector control and is likely to reach fixation soon, if pyrethroid use in this region is continued. These results demonstrate the strong selection pressures exerted on this allele by pyrethroid use. Our data also suggest, although further evidence is needed, that L1014S does not provide the same level of protection against pyrethroids as L1014F because L1014F appears to outcompete L1014S in *An. gambiae* populations that have been exposed to pyrethroids.<sup>49,50</sup> Based on previous studies, the presence of the *kdr* alleles has almost certainly had a detrimental impact on the efficacy of the vector control, and the incorporation of carbamates in later spray rounds can be considered prudent, given that no *ace-1* alleles were detected. Because of reports that *kdr* in combination with metabolic resistance can provide high levels of resistance, future vector control efforts in the country should include monitoring of metabolic resistance.

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