

Associated patterns of insecticide resistance in field populations of malaria vectors across Africa

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The development of insecticide resistance in African malaria vectors threatens the continued efficacy of important vector control methods that rely on a limited set of insecticides. To understand the operational significance of resistance we require quantitative information about levels of resistance in field populations to the suite of vector control insecticides. Estimation of resistance is complicated by the sparsity of observations in field populations, variation in resistance over time and space at local and regional scales, and cross-resistance between different insecticide types. Using observations of the prevalence of resistance in mosquito species from the *Anopheles gambiae* complex sampled from 1,183 locations throughout Africa, we applied Bayesian geostatistical models to quantify patterns of covariation in resistance phenotypes across different insecticides. For resistance to the three pyrethroids tested, deltamethrin, permethrin, and λ -cyhalothrin, we found consistent forms of covariation across sub-Saharan Africa and covariation between resistance to these pyrethroids and resistance to DDT. We found no evidence of resistance interactions between carbamate and organophosphate insecticides or between these insecticides and those from other classes. For pyrethroids and DDT we found significant associations between predicted mean resistance and the observed frequency of *kdr* mutations in the *Vgsc* gene in field mosquito samples, with DDT showing the strongest association. These results improve our capacity to understand and predict resistance patterns throughout Africa and can guide the development of monitoring strategies.

deltamethrin | permethrin | insecticide resistance genes | cross-resistance | *kdr*

Malaria prevalence in Africa has declined over the last 20 y and these gains have, in large part, been due to the widespread implementation of insecticide-based vector control measures (1). In the same time period we have also seen increases in insecticide resistance that have the potential to derail or even reverse the progress made in reducing malaria transmission (2, 3). It is therefore vital to understand the impact of insecticide resistance on malaria transmission.

Only 12 available insecticides, all insect neurotoxins, have WHO Pesticide Evaluation Scheme (WHOPES) approval for use in malaria vector control: 6 of these for insecticide-treated nets (ITNs), 3 for long-lasting ITNs (LLINs), and 11 for indoor residual spraying (IRS) (4). To steward this limited resource, WHO encourages countries to develop insecticide resistance management plans based on local data; however, these data are sparse or non-existent in many places. Insecticide resistance is often considered simplistically as a binary (dead or alive) response to insecticide exposure, but the resistance phenotype can be generated by a plethora of different mechanisms resulting in populations that differ in the spectrum and level of resistance to different insecticides. Moreover, resistance can vary temporally and spatially within and between insecticides and species. To understand the operational significance of resistance, we must consider the patterns of variation in resistance to each insecticide recommended for use. Understanding cross-resistance between insecticides, especially across

different classes, is also crucial for developing effective insecticide resistance management strategies.

Studies of the underlying mechanisms of resistance suggest that common patterns of phenotypic resistance to different insecticides may occur in field populations of mosquitoes. However, the quantitative impact of combinations of mechanisms found in the field remains poorly understood. Three categories of mechanism confer resistance to neurotoxic insecticides in malaria vectors: alterations to metabolic genes or pathways, target site mutation, and cuticular thickening. Metabolic resistance resulting primarily from the amplification or up-regulation of detoxification enzymes (especially esterases, P450 monooxygenases, and glutathione *S*-transferases) occurs commonly and can confer high levels of resistance (5–7). Some enzymes have been linked to resistance to a specific insecticide or class (8, 9) whereas others confer resistance across insecticide classes (5, 7, 10). Changes to insecticide target sites are also prevalent and frequently associated with phenotypic resistance (11–13). Acetylcholinesterase target site alteration caused by a mutation to the *Ace-1* gene typically confers cross-resistance to organophosphate and carbamate insecticides (5, 14). Cross-resistance to pyrethroids and the organochlorine DDT arise from knock-down resistance (*kdr*) mutations in the *Vgsc* gene which encodes the *para* voltage-gated sodium channel target site (15). There is currently less evidence for cuticular thickening as a mechanism of resistance (16–18).

Significance

Malaria control programs rely on chemical insecticides to target mosquito vectors and are potentially threatened by the emergence of insecticide resistance in African vector populations. Insecticide resistance management initiatives require comprehensive quantification of resistance in field populations to the set of insecticides used in vector control. We analyzed patterns of variation and covariation in resistance to these insecticides, using statistical methods that handle the sparse spatiotemporal distribution of the available data. We found relationships across different insecticide types that are consistent across large parts of Africa, allowing prediction of resistance to be improved by incorporating observations across multiple insecticide types. We also found large-scale relationships between phenotypic resistance and patterns of genetic variation, demonstrating the potential utility of genetic markers.

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Resistance to multiple insecticides within a chemical class is commonly noted but rarely quantified and differences in resistance to different insecticides within a class are also seen (13). Detection of resistance relationships is impeded by the high measurement error of discriminating dose bioassays that estimate the prevalence of resistant phenotypes. Additional complications arise from the co-occurrence, but uncertain impacts, of multiple mechanisms of resistance within mosquito populations and within individual mosquitoes (12, 19–21) and by poor understanding of the influence of environmental factors (22). We do not yet know how these varied resistance mechanisms—with their differing geographical distributions (23–25)—combine to impact variation in resistance within and among field populations or whether the cross-resistance patterns seen in laboratory studies translate into common geospatial patterns of phenotypic resistance to different types of insecticide in the field. If predictable relationships between resistance across different insecticides in field populations can be found, this will improve our ability to assess and manage the impact of resistance on vector control.

To date, the majority of tests of phenotypic insecticide resistance conducted on African malaria vectors have used *Anopheles gambiae* complex samples. Using data on the prevalence of insecticide resistance in samples collected from 1,183 locations in 38 African countries, we applied a Bayesian geostatistical modeling approach to analyze the relationships in resistance across the insecticides most commonly used in malaria vector control. For insecticides within the pyrethroid and organochlorine classes, we found consistent relationships in the prevalence of resistance across different insecticide types over a large part of sub-Saharan Africa. Making use of relatively abundant data on the frequency of well-characterized mutations in the *Vgsc* gene in field-collected samples from the *A. gambiae* complex, we demonstrated clear associations between the average prevalence of the resistance phenotype for DDT and pyrethroid insecticides and the frequency of *Vgsc* mutations.

These results develop our capacity to understand and predict phenotypic resistance patterns throughout Africa for commonly used insecticides.

Results

Large-Scale Resistance Phenotype Relationships Across Insecticides.

We investigated associations between resistance phenotypes across different types of insecticides using the results of WHO susceptibility bioassays conducted on mosquito samples from sub-Saharan Africa over the period 2000–2015 (26), using the proportional sample mortality from the bioassay. The distribution of the sample collection locations is heterogeneous across space and time and varies across the different insecticides tested (Fig. 1 *A–E* and *SI Appendix*, Figs. S1–S4). To accommodate this sampling heterogeneity we applied a Bayesian geostatistical modeling approach. Due to the lack of samples from central Africa, we first partitioned the data into two separate spatial regions covering western and eastern parts of the continent (Fig. 1 *A–D*). We then assessed resistance associations across different insecticide types by comparing a linear model of coregionalization (LMC) that allows interactions between resistances across each insecticide type with a model where resistances to the different insecticides are assumed to be independent. Two measures were used to compare the models: (i) the Watanabe–Akaike information criteria (WAIC) (27, 28) and (ii) the root-mean-square error (rmse) obtained from *K*-fold out-of-sample validation, calculated separately for each insecticide type. We performed independent model fits for the data subsets within the West and East spatial regions (*Materials and Methods*).

Associations within an insecticide class. For pyrethroids, we jointly model resistance observations for the three most commonly tested pyrethroids: deltamethrin, permethrin, and λ -cyhalothrin (Fig. 1). A LMC that allowed interactions between resistances across all three insecticide types performed better than a model

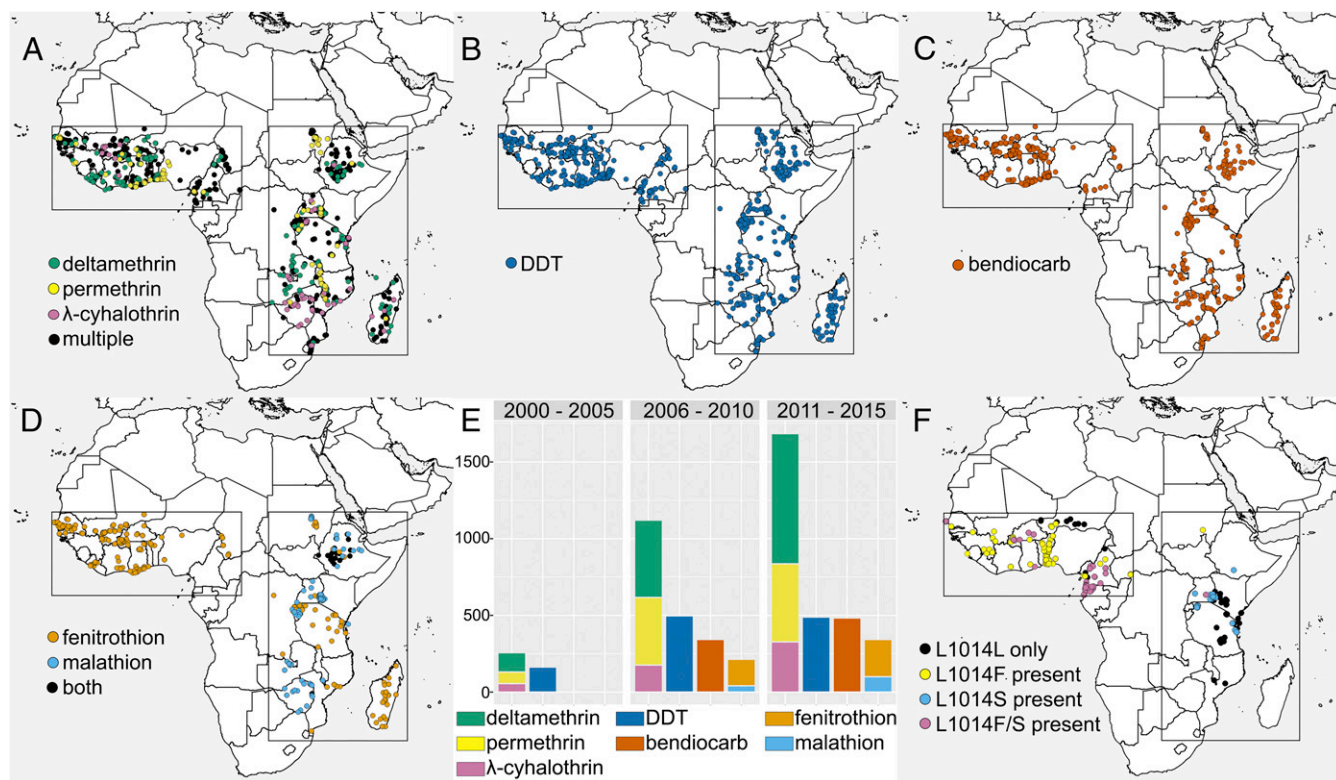


Fig. 1. The spatiotemporal distribution of the sample collection locations for insecticide resistance bioassays included in our dataset. Rectangles enclose the West and East regions considered in our analysis. (A) Pyrethroid (Py) bioassays. (B) Organochlorine (Och) bioassays. (C) Carbamate (Ca) bioassays. (D) Organophosphate (Oph) bioassays. (E) The number of bioassay records for each time period. The keys in A–D correspond to the insecticides shown in E. (F) The locations of sample collection used to calculate *Vgsc* allele frequency data. The mutations L1014F and L1014S present at each location are shown.

Table 1. The difference in the WAIC between a LMC and a model where resistances do not interact across different insecticides

Insecticide included in model	Δ WAIC (SE)	
	West region	East region
D, P, L	−134.2 (22.7)	−154.0 (28.3)
Och, D, P, L	−110.8 (18.3)	−44.3 (16.9)
Ca, D, P, L	5.5 (4.3)	−3.0 (11.9)
Oph, D, P, L	32.5 (16.7)	−4.7 (3.6)
Och, Ca	−4.2 (5.9)	0.7 (0.4)
Och, Oph	7.9 (5.5)	−0.17 (1.1)
Ca, Oph	−9.9 (19.5)	8.1 (5.7)

A negative Δ WAIC value indicates that the LMC performs better and a positive value indicates that a model with no interactions across insecticides performs better. We consider Δ WAIC values that are lower in magnitude than (or approximately equal to) the SE to be inconclusive. Substantial differences are highlighted in boldface type. Independent models are fitted to the West and East regions. The insecticide bioassays included in the models are denoted as follows: bendiocarb (Ca), DDT (Och), deltamethrin (D), λ -cyhalothrin (L), permethrin (P), and organophosphates including fenitrothion for the West region and both fenitrothion and malathion for the East region (Oph).

where these resistances did not interact according to both the WAIC (Table 1, row 1 and *SI Appendix*, Fig. S10) and the out-of-sample rmse (*SI Appendix*, Table S2, row 1). This indicates that associations between insecticides explain variation in the resistance observations. The predictions of the mean proportional mortality across each location and time show strong associations between the prevalences of resistance to the three insecticides in populations from both West and East Africa (Fig. 2). The Pearson correlation coefficient r between predicted prevalence of resistance to deltamethrin and that to permethrin has a posterior mode of $r_m = 0.91$ [credible interval (CI) = 0.90, 0.93] and $r_m = 0.94$ (CI = 0.94, 0.95) for the West and East regions, respectively. For both regions, predicted prevalence of resistance to permethrin is typically higher than that to deltamethrin, but with a greater difference apparent in West Africa (Fig. 2A). Among cyano-pyrethroids, higher prevalence of resistance to λ -cyhalothrin than to deltamethrin is seen in both West and East Africa [Fig. 2B; $r_m = 0.99$ (CI = 0.99, 1.0) for the West region and $r_m = 0.98$ (CI = 0.980, 0.984) for the East region]. The predicted resistance relationship between permethrin and λ -cyhalothrin is approximately linear for both regions, although the East region shows stronger relative resistance to λ -cyhalothrin in areas with high resistance to both insecticides [Fig. 2C; $r_m = 0.90$ (CI = 0.89, 0.92) for the West region and $r_m = 0.94$ (CI = 0.94, 0.95) for the East region]. Importantly, despite minor differences, the three predicted resistance relationships are similar between the West and East regions, and there was no credible difference between the two regions in the parameters describing the strength of the interactions across insecticides (*SI Appendix*, Fig.

S13). This indicates that phenotypic resistance associations across different pyrethroids are relatively homogenous across sub-Saharan Africa.

Associations across insecticide classes. We investigated associations between resistance to DDT and to pyrethroids by extending the LMC developed for the three pyrethroid types to include interactions between DDT and pyrethroids. This LMC performed better than a model where resistance to DDT did not interact with resistance to pyrethroids according to both the WAIC (Table 1, row 2) and the out-of-sample validation based on withheld DDT resistance observations (*SI Appendix*, Table S2, row 2). Predicted prevalence of resistance to DDT is much higher than that for the three pyrethroids (Fig. 3). The form of the relationships is similar between the West and East regions, although for the latter there are more outliers for which resistance to pyrethroids is higher (Fig. 3 and *Discussion*). Correlations between predicted prevalences of resistance to DDT and to pyrethroids are lower for the East than for the West African region. For the West region the correlations between prevalences of resistance to DDT and permethrin, deltamethrin, and λ -cyhalothrin were $r_m = 0.91$ (CI = 0.9, 0.92), $r_m = 0.91$ (CI = 0.89, 0.92), and $r_m = 0.92$ (CI = 0.91, 0.93), respectively. For the East region these correlations were $r_m = 0.65$ (CI = 0.61, 0.7), $r_m = 0.56$ (CI = 0.51, 0.6), and $r_m = 0.6$ (CI = 0.56, 0.64), respectively. The predicted strength of the interaction between DDT and pyrethroids was also greater for the West compared with the East region (*SI Appendix*, Fig. S14A).

We used the same approach to investigate associations between resistance to bendiocarb and pyrethroids. There was no evidence that a LMC that included interactions between resistance to the two insecticide classes performed better than a model that excluded these interactions (Table 1, row 3; *SI Appendix*, Table S2, row 3; and *SI Appendix*, Fig. S15). In other words, statistical evidence for broad-scale carbamate-pyrethroid associations was absent for this pair of classes, despite local evidence from *Anopheles coluzzii* for cross-acting metabolic mechanisms (5). We also assessed associations between resistance to organophosphates and resistance to pyrethroids, using a modified LMC for the East region to account for the two different organophosphate insecticides, fenitrothion and malathion [*Materials and Methods*; we note that due to the small number of available observations, we did not assess relationships in resistance between these two organophosphate insecticides (Fig. 1)]. There was no evidence that the LMCs performed better than models that excluded interactions between resistance to organophosphates and resistance to pyrethroids (Table 1, row 4 and *SI Appendix*, Table S2, row 4); i.e., once again broad-scale resistance associations were absent (e.g., *SI Appendix*, Fig. S16). Finally we investigated relationships between resistance across the remaining pairs of insecticide classes, including organophosphate-bendiocarb, organophosphate-DDT, and bendiocarb-DDT resistance associations, using LMCs with only one interaction term. In all cases, our model comparison did not support choosing the LMC over a model that excluded interactions between resistance across different insecticide classes (Table 1 and *SI Appendix*, Table S2, bottom three

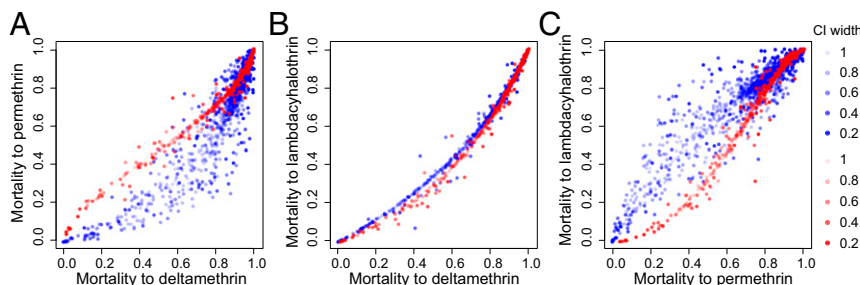


Fig. 2. Relationships between predicted mean proportional bioassay mortalities for the three pyrethroid types. (A) Deltamethrin vs. permethrin. (B) Deltamethrin vs. λ -cyhalothrin. (C) Permethrin vs. λ -cyhalothrin. Points show the predicted mean at each location and time for the West region (blue) and the East region (red). Color intensity indicates the width of the posterior CI of the predicted mean.

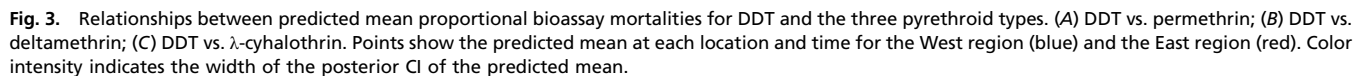


Fig. 4. The mortality to DDT predicted by a fitted linear relationship with observed *Vgsc* allele prevalences ($n = 316$). Estimates of mean mortality following exposure to DDT obtained from our Bayesian geostatistical models (Fig. 3) were used as the response variable. Color intensity indicates the relative posterior precision of the mean mortality estimates. The diagonal line indicates equivalence between the data and the fitted values.

insecticides were included. This was unexpected because mutations in the *Ace-1* gene are known to confer resistance to both organophosphates and carbamates where present in West Africa (5, 14). This result may reflect the lower power to detect associations for these classes because data volumes are lower (*SI Appendix, Table S1*) and perhaps most importantly, variation in mortality was less, especially for the organophosphates to which resistance remains limited. In addition, while organophosphate insecticides were pooled for this analysis, cross-resistance between insecticides within this class has been found to be variable, which could cause variable cross-class interactions in field populations (30–32).

Our results demonstrate broad-scale field population-based associations between *Vgsc* allele frequency and phenotypic resistance to DDT and pyrethroids. Local studies of the influence of different *Vgsc* alleles on levels of resistance in field populations have not shown consistent results (33), but our findings provide evidence that *Vgsc* allele frequencies are a useful partial diagnostic for resistance. Our findings are consistent with genetic studies that have shown that *Vgsc* mutations have emerged and spread as insecticide resistance has emerged and spread (10, 23–25, 34). The association found here is strongest with DDT resistance, which is consistent with field studies that have found stronger links between *Vgsc* alleles and DDT resistance compared with pyrethroid resistance (13, 35) and stronger relationships between metabolic resistance enzymes and pyrethroid resistance compared with DDT resistance (36, 37). Importantly, our results demonstrate the potential utility of genetic markers. Given an appropriate sampling procedure, use of DNA diagnostics to predict resistance phenotypes could provide a means of increasing resistance data resources to support monitoring programs.

An important limitation of our results is that they consider resistance in the *A. gambiae* species complex as a whole. The available bioassay data are predominately linked to the species complex because live mosquitoes are required and sibling species can be distinguished only by molecular analysis. As noted above, individual species are known to differ in the underlying mechanisms of resistance, phenotypic levels of resistance, and their geographical distributions. These factors can be partitioned only when sufficient single-species data are available for analysis, and we advocate, wherever possible, that field studies should type the mosquitoes tested.

In this study, we have considered resistance to seven insecticides of importance to current malaria vector control. In addition, pirimiphos-methyl, α -cypermethrin, bifenthrin, cyfluthrin, and etofenprox are approved and available for use (4). Despite the call by WHO in 2012 for countries to undertake routine resistance monitoring to support operational activities (38), the combined pan-African dataset for 2000–2015 is still too sparse to assess resistance associations comprehensively across the full array of insecticides available for malaria control. Due to the high variability of resistance prevalence across the *A. gambiae* complex and the measurement error associated with susceptibility bioassays, a comprehensive spatial coverage of samples, as well as replicate sampling, is required to robustly assess variation in resistance phenotypes and any associations with genetic variation.

In conclusion, despite regional variation in species and specific resistance mechanisms, we have demonstrated clear broad-scale associations in the average prevalence of resistance among the pyrethroids and between the pyrethroids and DDT and provided a verification of *Vgsc* mutations as useful predictors of DDT and pyrethroid resistance. Associations across pyrethroids have the potential to improve our predictions of the prevalence of resistance to insecticides within this class. In resource-limited environments, testing a single pyrethroid insecticide can provide control programs with useful estimates of resistance to other pyrethroids. However, caution is needed because there is uncertainty in these predictions and where resources are sufficient the best approach is still to conduct resistance monitoring for all insecticides being used for malaria control, or being considered for future control, at multiple locations (39).

Materials and Methods

Insecticide Resistance Bioassay Data. Insecticide resistance bioassay data were obtained from the collated data for bioassays conducted on mosquito samples collected globally described by Coleman et al. (26). The data record the number of mosquitoes in the sample and the proportional sample mortality resulting from the bioassay, as well as information about the mosquitoes tested, the sample collection site, and the bioassay conditions and protocol (26). This information was used to select a subset of records for inclusion in our study (*SI Appendix, Figs. S1–S4*). In summary, we consider only bioassays conducted using standard WHO susceptibility tests on mosquito samples belonging to the *A. gambiae* complex, conducted over the period 2000–2015 on samples collected within two separate spatial regions selected because they had sufficient data (Fig. 1). The final dataset contained a total of 5,595 data points (*SI Appendix, Table S1*) but does not provide values for all of the insecticides of interest at each of the 1,183 locations so associations among these insecticides could not be analyzed directly. Further, the spatiotemporal distribution of data for each insecticide is not uniform or random and each distribution incorporates sampling biases (Fig. 1 and *SI Appendix, Figs. S1–S4*). For these reasons we evaluated the relationships among the insecticides by incorporating these datasets from field populations into a geostatistical model. We found that performing independent model fits to each separate spatial region improved prediction accuracy (see *SI Appendix, section S3* and the next section for further explanation of how this was assessed).

Spatiotemporal Bayesian Statistical Models. We assess resistance associations across different insecticide types using a Bayesian geostatistical model that extrapolates predictions across space and time by estimating the spatiotemporal correlation structure in the observations. Let $y_A(s_i, t)$ denote the proportional mortality record for a bioassay using insecticide type *A* conducted on a sample collected at geographic coordinates s_i and sampling time t . To improve posterior validation (assessed using the methods described below) we applied two transformations to these observations, the empirical logit transformation followed by the inverse hyperbolic sine transformation (40). We then assumed that these transformed observations, denoted $g_A(s_i, t)$, arise from a spatiotemporal process governed by the measurement equation

$$g_A(s_i, t) = \beta_A + u_A(s_i, t) + e_A, \quad [1]$$

where β_A is a constant/intercept, $u_A(s_i, t)$ is a Gaussian process modeled as a spatiotemporal Gaussian Markov random field (GMRF) (1, 41, 42), and e_A is Gaussian white noise $N(0, \sigma_A^2)$. To jointly model bioassay mortality observations for two insecticides *A* and *B* where the observation locations and times differ between the two insecticides, we used a LMC (41, 43, 44). The model of the logit-transformed mortality observations for insecticide *B* is then given by

$$g_B(s_i, t) = \beta_B + \lambda_{B,A} u_A(s_i, t) + u_B(s_i, t) + e_B, \quad [2]$$

where $\lambda_{B,A}$ is a constant coefficient and β_B , $u_B(s_i, t)$, and e_B are defined in the same way as above, replacing *A* with *B* in the subscript. We define a Bayesian hierarchical formulation for the joint model given by [1] and [2], using a vector of prior probability distributions π for the hyperparameters $\theta = [\beta_A, \beta_B, \sigma_A, \sigma_B, \psi_A, \psi_B, \lambda_{B,A}]$, where ψ_A and ψ_B are the parameters of $u_A(s_i, t)$ and $u_B(s_i, t)$ (*SI Appendix, section S6*). We implement these models using the R-INLA package (www.r-inla.org) to obtain estimates of the posterior distributions of $u_A(s_i, t)$, $u_B(s_i, t)$, and θ (45, 46). To quantify the association between resistance across insecticide types *A* and *B* we draw 1,000 simulations from the posterior distribution of the mean mortality for each insecticide type, $\bar{y}_A(s_i, t)$ and $\bar{y}_B(s_i, t)$, across a set of locations j that are defined by the mesh nodes of the GMRFs $u_A(s_i, t)$ and $u_B(s_i, t)$ (43). For each posterior draw we calculate the Pearson correlation coefficient, r , between the predicted mean mortality values for each insecticide type and estimate the posterior distribution of r based on all posterior draws. We use similar LMC methods to jointly model bioassay mortality observations for up to four different insecticide types *A*, *B*, *C*, and *D* (*SI Appendix, section S6* and ref. 44).

We compare performance of these LMCs that allow interactions between resistances across each insecticide types to the performance of models that assume independence of bioassay mortality across different insecticide types to assess whether a joint model best predicts the patterns of resistance seen in field populations. Independence across insecticides is modeled by using Eq. 1 to represent proportional mortality for all insecticides, replacing *A* by *B*, *C*, or *D* in the subscript. To compare the predictive accuracy of the two model types we compare the WAIC, which use the full posterior distribution of the predicted parameters (27, 28). We also calculated the SE of the difference in the pointwise WAIC values of each model to give an indication of the uncertainty associated with the difference in the WAIC (47) (*SI Appendix, Fig. S10*). For the organophosphates class where we have a small number of

resistance observations for each insecticide type, we use a modified version of Eq. 1 that assumes the same pattern of spatiotemporal variation in resistance for both insecticides but allows resistance to each insecticide to differ by a fixed effect. We perform posterior validation using posterior predictive checks, probability integral transform (PIT) histograms (on out-of-sample data), and 10-fold out-of-sample cross-validation (*SI Appendix, section S2*).

Vgsc Allele Prevalence Data. We obtained data on the prevalence of *Vgsc* mutations in mosquito samples from the dataset collated by Coleman et al. (26), which describes observed frequencies of point mutations in the *Vgsc* gene. We extracted those records that reported the proportion of different *Vgsc* alleles in *A. gambiae* complex samples collected within the West and East regions of Africa (Fig. 1) over the period 2005–2015. Although some of these data report individual genotypes, we use these to derive the *Vgsc* allele frequency, because many of the studies reported only allele frequencies. We then applied a process of screening the data to exclude potentially biased estimates of *Vgsc* allele frequency (*SI Appendix, section S7*). This conservative approach resulted in a reduction in data volume from 1,429 records to 316, incorporating 215 and 101 observations for the West and East regions, respectively (Fig. 1).

Modeling Associations Between Genotype and Phenotype Prevalence Across Populations. We used OLS linear regression to assess associations between the predicted mean mortality following exposure to a given insecticide type and the prevalence the two *Vgsc* mutations, L1014F and L1014S, across the genotyped mosquito samples described above. Values of the predicted mean mortality were obtained from our fitted geostatistical models (Figs. 2 and 3) and were represented on the scale of the transformations applied to the insecticide resistance bioassay data described above. Before model fitting we applied the empirical logit transformation to the *Vgsc* mutation prevalence observations. We verified the normality of the residuals, using the Shapiro–Wilk test ($P = 0.154$). Examination of the residuals revealed contemporaneous spatial correlation, so we performed inference on the regression coefficients using cluster-robust SEs (48, 49), setting the cluster radius to 100 km (*SI Appendix, section S5*).

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