OXFORD

Potential Roles of Environmental and Socio-Economic Factors in the Distribution of Insecticide Resistance in *Anopheles gambiae sensu lato* (Culicidae: Diptera) Across Togo, West Africa

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Subject Editor: Raul Narciso Guedes

Abstract

Vector control strategies recommended by the World Health Organization are threatened by resistance of *Anopheles* mosquitoes to insecticides. Information on the distribution of resistant genotypes of malaria vectors is increasingly needed to address the problem. Ten years of published and unpublished data on malaria vector susceptibility/resistance and resistance genes have been collected across Togo. Relationships between the spatial distribution of resistance status and environmental, socio-economic, and landscape features were tested using randomization tests, and calculating Spearman rank and Pearson correlation coefficients between mosquito mortality and different gridded values. *Anopheles gambiae sensu lato* was resistant to DDT, pyrethroids, and the majority of carbamates and organophosphates. Three sibling species were found (i.e., *An. gambiae*, *Anopheles coluzzii*, and *Anopheles arabiensis*) with four resistance genes, including kdr (*L1014F*, *L1014S*, and *N1575Y*) and *ace1* (*G119S*). The most frequent resistance gene was *L1014F*. Overall, no association was found between the susceptibility/resistance status and environmental features, suggesting that evolution of resistance may be most closely related to extreme selection from local insecticide use. Nevertheless, further research is necessary for firm conclusions about this lack of association, and the potential role of landscape characteristics such as presence of crops and percentage of tree cover.

Key words: malaria vector, susceptibility/resistance status, resistance gene, climatic feature, raster data

Malaria vector control strategies recommended by the World Health Organization (WHO), namely use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS; WHO 2018a), have been facing problems with resistance in the last few years (WHO 2016). Insecticide resistance is widespread in Sub-Saharan African countries, particularly in West Africa, where pyrethroid resistance is of main concern (WHO 2018b). Indeed, it was reported that in most cases, mortality rates in malaria mosquitoes subjected to pyrethroid-impregnated papers were below 90% (WHO 2018b), the current WHO resistance threshold (WHO 2013). This resistance derives mainly from genetic mutations (Hancock et al. 2018) and enzyme over-expression (Matowo et al. 2010).

Resistance selection in malaria vectors has been attributed to excessive use of insecticides in agriculture, but also to anthropogenic or natural xenobiotics present in mosquito breeding sites (Diabaté et al. 2002, Nkya et al. 2013). Agriculture comprises 70% of the economy of Togo, and, depending on the crop cultivated, the area, and the nature of the soil, different agricultural practices are adopted by the local population (Agboyi et al. 2015); insecticides and other agrochemicals are used in this context to control pests. Given excessive use, insecticide resistance in malaria vectors has also been reported in the country (Ahadji-Dabla et al. 2014). Nevertheless, a crucial question arises: are those practices the only factors affecting

the distribution of the resistance/susceptibility of *Anopheles* mosquitoes to insecticides across the country, or is the distribution of resistance modulated by environment?

Environmental variables (e.g., temperature, humidity, vegetation, etc.) are known to play key roles in mosquito biology by influencing their distribution and abundance (Muturi et al. 2008). Several studies have documented the distribution of *Anopheles* species in relation to climate features (Levine et al. 2004, Kelly-Hope et al. 2009, Peterson 2009, De Sousa et al. 2010, Siraj et al. 2014), but less is known about influences of environmental factors on resistance mechanisms in *Anopheles* populations. In Togo, three malaria vectors (i.e., *Anopheles coluzzii*, *An. gambiae*, and *Anopheles arabiensis*) belonging to the *Anopheles gambiae* complex have been reported in different agro-ecosystems (Amoudji et al. 2019), and are known to be resistant to insecticides, mostly to DDT and pyrethroids. Venter et al. (2017) stated that information on the distribution of malaria vector species and their susceptibility profiles is needed for development of an effective vector control program. This study, therefore, aimed to 1) provide information about susceptibility and resistance status of *An. gambiae sensu lato* populations across Togo, 2) assess genetic resistance alleles of each sibling species of the complex, and 3) examine the spatial distribution of susceptible/resistant mosquitoes and its relationship with environmental, socio-economic, and landscape features in Togo. Our aim was to assemble the most comprehensive set of studies of resistance status of these mosquitoes across Togo, to permit in-depth analysis of external correlates of resistance.

Materials and Methods

Study Site

Togo is located in West Africa, with Burkina Faso to the north, Ghana to the west, and Benin to the east (Fig. 1). The surface area is 56,600 km², with an approximate population of 7 million inhabitants. Togo enjoys a tropical climate with two rainy seasons per year, from April to June and from September to October, in the southern half of the country; annual precipitation ranges 800–1,400 mm. The northern half of the country sees only one rainy season, from June to September. Mean annual temperature is 27°C (MERF 2015). In terms of ecology, Togo is divided into five zones (Ern 1979): the plains and mountains zones to the north (zones I and II, respectively), the central plains area that is zone III, the Mounts Togo meridional zone (zone IV), and zone V in the south that includes the coastal plain. Cereals and tubers are the main crops grown in zones I (Dapaong, Tandjouaré, and Mango) and III (Efofami-Yéyé, Kolokopé, and Nangbéto), cereals in zone II (Siou, Tchitchao), cereals, cocoa, and coffee in zone IV (Kpélé, Lom Nava), and rice and vegetable in zone V (Kovié, Agoè, Baguida, Akodesséwa, and Gbadago; Fig. 1).

Entomological and Polymerase Chain Reaction Genotyping Data Collection and Mapping

Various sources were consulted for data collection on the susceptibility/resistance of *An. gambiae s.l.* to insecticides and on resistance mechanisms covering 10 yr (2009–2018). This information includes doctoral and master's theses defended at the University of Lomé (Togo), reports of the National Malaria Control Programme (NMCP) of Togo, data from on-going studies by the authors, and articles published in peer-reviewed journals (Ahadji-Dabla et al. 2014, 2015, 2017; Djègbè et al. 2018). Information including locations of larval surveys, study periods, species and insecticides tested, mortality rates, and geographic coordinates (longitude and latitude) were obtained. Eight insecticides belonging to four classes were considered in this study: organochlorine (4% DDT), pyrethroids (0.75% permethrin and 0.05% deltamethrin), carbamates (0.4% carbosulfan and 0.1% bendiocarb), and organophosphates (5% malathion, 0.4% chlorpyrifos methyl, and 1% fenitrothion). In terms of the distribution of genetic resistance alleles in key species, knockdown resistance (*kdr*; L1014F, L1014S, and N1575Y) and insensitive acetylcholinesterase (*ace1*; G119S) alleles were considered.

Susceptibility bioassays were conducted according to the WHO standard protocol (WHO 1998, 2013). Polymerase chain reaction (PCR) analyses for species identification followed Scott et al. (1993) and Fanello et al. (2002). For *kdr* and *ace1* genotyping, PCR was performed following the methods of Martinez-Torres et al. (1998), Ranson et al. (2000), Weill et al. (2004), and Bass et al. (2007, 2010). Overall, information from 17 localities was obtained in this study (Fig. 1), with 13 accounting for susceptibility/resistance data collection (Fig. 2), and 10 testing for specific resistance alleles (Fig. 3).

Geographic coordinates from study localities were obtained with a geographic position system device (Garmin 48) in Universal Transverse Mercator (UTM) coordinates, and transformed to decimal degrees using Google Earth (http://www.google.com/earth/). Locality data were imported into QGIS 2.18.23 ('Las Palmas' version) for analysis and mapping (Supp Table 1 [online only]). Distributions of the susceptibility/resistance of *An. gambiae s.l*, to insecticides and the prevalence distributions of genetic resistance alleles of the three key sibling species across the country were mapped.

Susceptibility/Resistance Categories and Data Analyses

Climate information for each locality was obtained from the MERRAclim repository at 2.5′ (~4.5 km) resolution (Vega et al. 2017). These environmental raster layers are derived from satellite imagery and include 19 bioclimatic variables summarizing worldwide temperature and humidity values for different time periods. Layers from 2000 to 2010 corresponding to annual mean temperature (BIO1), maximum temperature of the warmest month (BIO5), temperature seasonality (BIO4), minimum temperature of the coldest month (BIO6), and annual mean specific humidity (BIO12), specific humidity of the most humid month (BIO13), specific humidity of the least humid month (BIO14), and specific humidity seasonality (BIO15), for a total of eight climatic variables (Table 1) (Vega et al. 2017) were used.

Because insecticide resistance is mostly considered an anthropogenic driven phenomenon, socio-economic data for the 13 localities with susceptible/resistance information using data layers summarizing human population counts and densities and gross domestic product (CIESIN 2018a, b; Kummu et al. 2018) were also explored. Further, as a proxy of landscape characteristics, satellite-derived images summarizing the presence/absence of croplands (Xiong et al. 2017), nighttime lights (Baugh et al. 2010), and percentage of tree cover (DiMiceli et al. 2017) were used; for these three products, a buffer of 10 km around each of the 13 localities was developed and the proportion of pixels with crops for the corresponding layer, and the mean values for nighttime lights and tree cover layers were calculated. Because Akodesséwa and Baguida are separated by ~5.5 km, a buffer of 10 km in each of them recovers almost half of the pixels of the other (Fig. 1), thus, for croplands, nighttime lights, and percentage tree cover, values were extracted twice, each time eliminating one of these localities. Finally, Human Footprint layer, which ranks the impact of human presence by encompassing the combined influence of eight gridded products including nighttime lights, population density, crops, among others
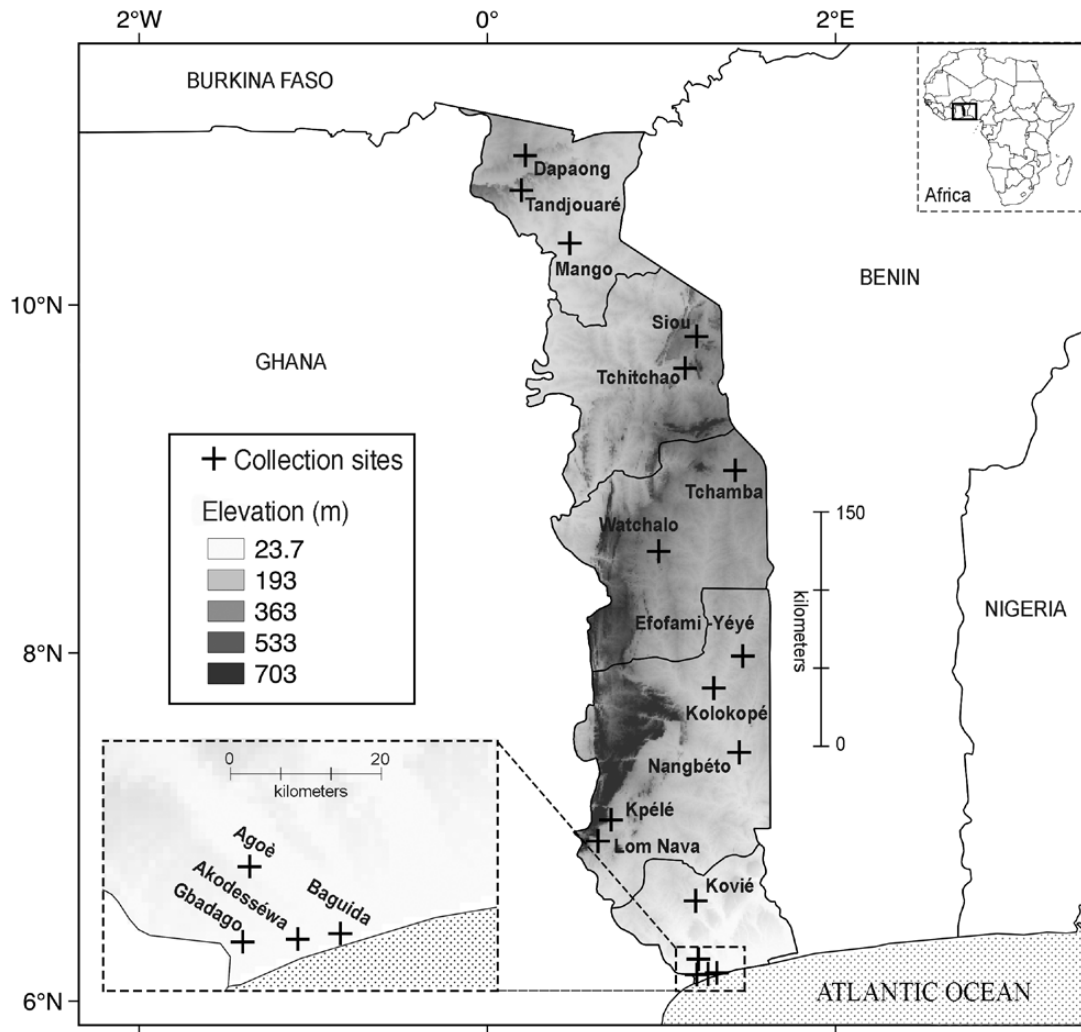
Fig. 1. Map of Togo, showing the collection sites analyzed in this study.

(Venter et al. 2016) was used. Spatial and temporal resolution, availability, and further description of each of the 15 variables used can be found in Table 1.

For each insecticide family, median *Anopheles* mortality rate was calculated considering mortality thresholds of 80 and 90% to determine level of mosquito susceptibility in each locality (WHO 1998, 2013; Supp Table 1 [online only]). To test for spatial associations with each of the selected variables, Monte Carlo analysis using the difference between the average values at susceptible versus resistant sites in each locality (or buffer) as an empirical observation of spatial associations (Gotelli and Ellison 2013) was performed. Then, 100,000 replicates were used, in which assignment to resistance categories was randomized to build a null distribution. An $\alpha = 0.05$ was used to determine whether the empirical observation was statistical different from the null (Gotelli and Ellison 2013). Also, Spearman rank and Pearson correlation coefficients were calculated between mosquito mortality and raster-derived values; as before, statistical significance was considered with $\alpha = 0.05$ (Gotelli and Ellison 2013). To account for the combined influence of the different climatic features, a principal component analysis (PCA) was performed and the same randomization and correlation tests as described above were developed, using the first (PC1), second (PC2), and third (PC3) components, which together accounted for 99.5% of the variance (Supp Figs. 1 and 2 [online only]).

Results

Relationship Between Susceptibility/Resistance and Environmental Associations

Susceptibility and resistance data were obtained for 13 localities. Resistance to organochlorine (i.e., DDT) and pyrethroids (i.e., permethrin and deltamethrin) was recorded in all sites (Fig. 2), except for susceptibility to 0.05% deltamethrin at Tchitchao (mortality = 82.1%, considering WHO 1998 criteria). Thus, spatial relationships of susceptible/resistant patterns could not be tested with randomization tests for lack of susceptible populations for these insecticides.

For carbamates, an 80% threshold showed high insect mortality at four sites Akodesséwa, 96.0%, Dapaong, 82.5%, Kolokopé, 89.9%, and Lom Nava, 96.8% (Fig. 2), and low mortality rates at the other nine sites. Randomization tests based on differences between the average values of susceptible and resistant sites yielded no significant relationship in any of the individual climatic variables or the PCs. The same negative results were obtained in comparisons using a 90% mortality threshold, although only two sites remained as susceptible: Akodesséwa and Lom Nava (Fig. 2, Supp Table 1 [online only]).

Socio-economic variables yielded similar negative results using either of the two thresholds: neither population nor gross domestic
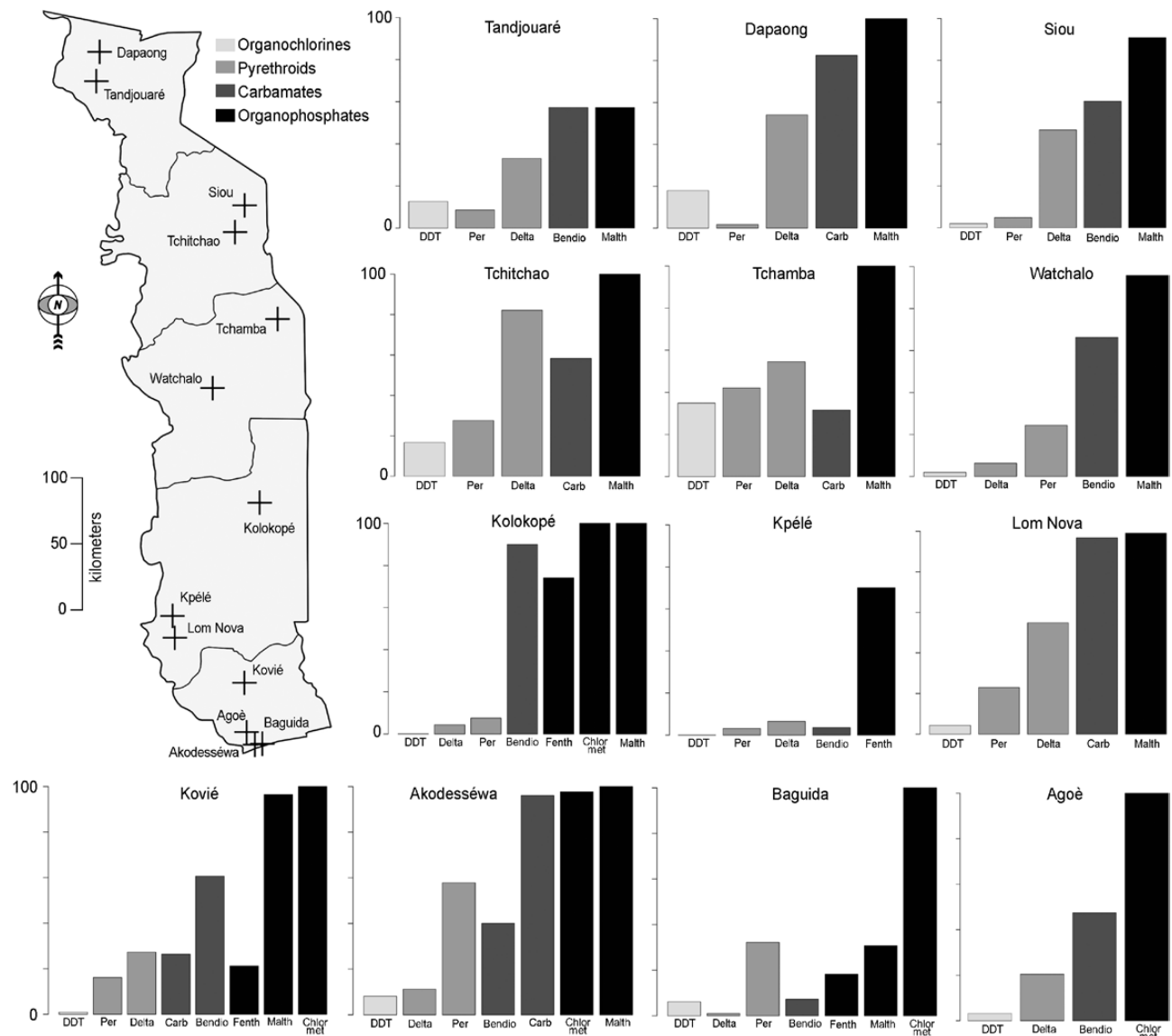
Fig. 2. Distribution of the susceptibility/resistance status of *Anopheles gambiae s.l.* DDT: 4% dichlorodiphenylchloroethane, Per: 0.75% permethrin, Delta: 0.05% deltamethrin, Carb: 0.4% carbosulfan, Bendio: 0.1 bendiocarb, Fenth: 1% fenitrothion, Chlormet: 0.4% chlorpyrifos methyl, and Mal: 5% malathion.

product explained carbamates resistance in the 13 localities explored. When applying the randomization test for landscape variables using the threshold at 90%, the presence of crops was positive when developing the test with 12 points, using Akodesséwa instead of Baguida (median susceptibility = 7.2%) for the analysis. However, the result was unstable, as the analysis developed using Baguida and eliminating Akodesséwa yielded a nonsignificant result (Supp Fig. 3 [online only]). A similar situation occurred when performing the analysis with a 90% resistance threshold and percentage of tree cover: analyses based on Baguida placed the empirical observation in 25th percentile of the null distribution, but this signal disappeared when repeating the analysis with Akodesséwa (Supp Fig. 3 [online only]). Repeating the analysis excluding both localities (*n* = 11), neither presence of crops nor percentage of tree cover was consistently positive. Analyses of human footprint index across our 13 localities showed consistently nonsignificant results.

For organophosphates, susceptible and resistant sites were the same with 80% or 90% mortality thresholds; low mortality sites included Kpélé (70.2%) and Tandjouaré (57.3%); Akodesséwa and

Baguida had mortality rates of 100% (Fig. 2). Randomization tests showed the empirical comparison to be no different from the null distribution in all climatic dimensions explored and in the PCs. Furthermore, randomization tests with the socio-economic, landscape, or human footprint variables showed no significant results. Nighttime lights empirical observation fell in the 25th percentile of the null distribution as in the case described above (Supp Fig. 3 [online only]).

Spearman rank and Pearson correlation coefficients were nonsignificant in all but three comparisons considering insecticides and each of the climatic, PCs, socio-economic, landscape, and human footprint variables. The Spearman rank correlation coefficient between DDT susceptibility and temperature of the warmest month (BIO5) was statistically significant (ρ = 0.65, P = 0.016). However, in this comparison, two sites showed no susceptibility (i.e., Kolokopé and Kpélé; mortality = 0%) and the highest mortality in Tchamba was 34.9%; hence, a linear regression model failed to show any significant relationship between the two variables (R^2 = 0.14, P = 0.11). Similarly, DDT susceptibility and nighttime
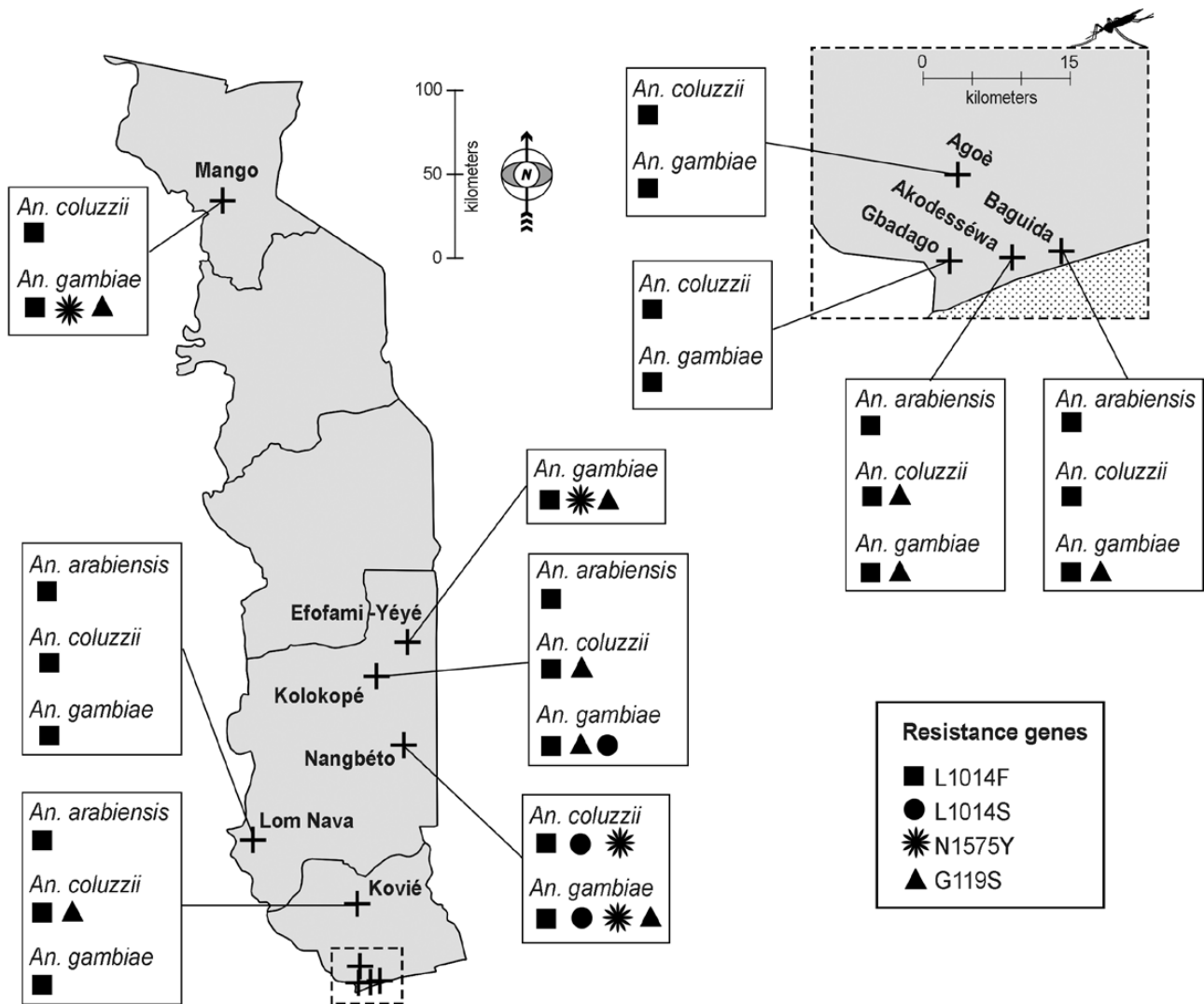
Fig. 3. Distribution of resistance genes in sibling species of *Anopheles gambiae s.l.* across Togo.

lights showed a significant correlation (ρ = 0.56, P = 0.04), but again a linear regression model failed to show the same statistical result (R^2 = –0.08, P = 0.73). The last significant Spearman correlation was found between organophosphate susceptibility and night-time lights (ρ = 0.62, P = 0.03); in this case, ten of the localities were susceptible and only two resistant (e.g., Kpélé = 70.19% and Tandjouaré = 57.25%), such that no significant linear regression could be fit (R^2 = 0.03, P = 0.33).

Species and Resistance Mutation Distribution

From 2009 to 2018, three sibling species of *An. gambiae s.l.* were identified: *An. coluzzii*, *An. gambiae*, and *An. arabiensis* (Fig. 3); hybrid species (*An. coluzzii*/*An. gambiae*) were identified at Baguida and Kovié but are not shown on the map. Four resistance mutation alleles were detected: kdr *L1014F* was the most common, and *L1014S*, *N1575Y*, and *ace1* were less frequent (Fig. 3). Knockdown *L1014F* allele, that was the most broadly represented in the country (eight localities), was present mostly in *An. coluzzii* and *An. gambiae*, but only rarely in *An. arabiensis* (five localities: Akodesséwa, Baguida, Kolokopé, Kovié, and Lom Nava). Additionally, *L1014S*, *N1575Y*, and *G119S* were detected in *An. gambiae* at Nangbéto (East Plateau Region), and *L1014S* and *G119S* were detected in *An.*

gambiae at Efofami-Yéyé (East Plateau Region) and Mango (North Savanna Region).

Discussion

The physiology of all organisms is in some sense and at some scale a function of environmental variables, which translates geographic distributions via the fundamental ecological niche. Change in mosquito distributions may reflect these effects; for instance, according to Peterson (2009), climate change effects on African malaria vectors (i.e., *An. gambiae s.s.* and *An. arabiensis*) will shift their distributional potential from West Africa increasingly to the east and farther south. Here, effects of environmental features (i.e., temperature and humidity) on the distributions of susceptible versus resistant *An. gambiae s.l.* genotypes were assayed with respect to insecticides. No correlation was found between susceptible versus resistant populations and the climatic variables analyzed in this study. Analyzing an extended set of variables summarizing coarse-grained dimensions of anthropogenic influence in our studied sites, a lack of association between socio-economic and human footprint factors was found. However, in the case of carbamate resistance at 90% threshold, presence of crops and percentage of tree forest recovered

Table 1. Raster products used for the spatial analysis (*n* = 15)

Variable category	Spatial / Temporal resolution / Description	Source / Reference	Availability
Temperature and humidity (Climate)	– 2.5 arc-minute / 2000–2010 / 1. Annual mean temperature (BIO1) 2. Temperature seasonality (BIO4) 3. Maximum temperature of the warmest month (BIO5) 4. Minimum temperature of the coldest month (BIO6) 5. Annual mean specific humidity (BIO12) 6. Specific humidity of most humid month (BIO13) 7. Specific humidity of least humid month (BIO14) 8. Specific humidity seasonality (BIO15)	MERRAclim (Vega et al. 2017)	https://datadryad.org//resource/doi:10.5061/dryad.s2v81
Population (Socio-economic)	– 2.5 arc-minute / 2015 / 1. Gridded population of the World version 4 (GPW v4): Population density. Adjusted to Match 2015. Revision of United Nations (UN). World Population Prospects (WPP) 2. GPW v4: Population counts Adjusted to Match 2015. Revision of UN. WPP	Center for International Earth Science Information Network-CIESIN-Columbia University 2018	http://sedac.ciesin.columbia.edu/data/set/gpw-v4-population-density-adjusted-to-2015-unwpp-country-totals-rev11
Gross domestic product (Socio-economic)	– 5 arc-minute / 2015 / 1. Gridded gross domestic product (GDP). Derived from GDP Purchasing Power Parity (PPP) per capita	(Kummu et al. 2018)	https://datadryad.org/resource/doi:10.5061/dryad.dk1j0
Croplands (Landscape)	– 30 arc-second / 2015 (re-scaled) / 1. Global Food Security-support Analysis Data (GFSAD) version 001. Cropland extent Africa 30 m (original resolution)	(Xiong et al. 2017)	https://e4ftl01.cr.usgs.gov/MEASURES/GFSAD30AFCE.001/2013.01.01/
Vegetation Continuous Fields (VCF) (Landscape)	– 30 arc-second / 2010 (re-scaled) / 1. MOD44B version 006. Percentage of tree cover at 250 m (original resolution)	VCF-LP DAAC-MOD44B	https://e4ftl01.cr.usgs.gov/MOLT/MOD44B.006/
Nighttime lights (Landscape)	– 30 arc-second / 2013 / 1. DMSP-OLS Nighttime Light. Time Series version 4. Stable lights product	(Baugh et al. 2010)	https://ngdc.noaa.gov/eog/dmsp/downloadV4composites.html#AVSLCFC
Human footprint (Environmental impact)	– 30 arc-second / 2009 / 1. Composite HFT-2009 product. Ensemble of eight cumulative ranked variables of information including: buildings, population density, nighttime lights, croplands, pasture, roads, railways, and navigable waterways	(Venter et al. 2016)	https://datadryad.org/resource/doi:10.5061/dryad.052q5

For each of the 13 localities we extracted the values corresponding to their respective pixel. For croplands, nighttime lights, and percentage tree cover we extracted values from a buffer of 10 km from the occurrence point. Re-scaled refers to croplands and vegetation continuous fields that were up-scaled from 30 and 250 meters respectively to 30 arc-second resolution.

ambiguous results when including or excluding either Akodesséwa or Baguida (Supp Fig. 3 [online only]). The methodology used could be more efficient and would have more statistical power if larger sample sizes were available; that is, the number of localities with susceptible/resistance information with which to perform the spatial comparisons was constrained (*n* = 13), and because susceptible/resistance categories were not distributed evenly (e.g., eight resistance vs. two susceptible localities for carbamates with a 90% threshold). Although the Monte Carlo randomization approach used here, is a relatively assumption-free test useful to deal with small sample size data; the negative results and preliminary potential associations should be interpreted in the context of the present study and the data available (Gotelli and Ellison 2013); such effects may be discernible only with larger sample sizes, especially for percentage of tree cover and crops, or with sites across broader and more environmentally diverse regions.

Many studies have reported that high metabolic rates allow animals to increase the range of environmental temperatures under which they can function and maintain homeostasis (Bozinovic et al. 2011). Such increases could influence their geographic distributions in terms of how they respond to xenobiotics like insecticides. According to some authors, mosquitoes set in motion their enzymatic arsenal (e.g., esterases, oxidases, transferases) to metabolize insecticides (Hemingway et al. 2004). This metabolism could be easily modulated by their environment, in turn influencing geographic

distributions. Thus, future research in this vein is encouraged before concluding a lack of association between environmental variables and insecticide resistance in Togo.

In addition to enzyme over-expression, target site mutation is among the resistance weapons used by *Anopheles* mosquitoes. In voltage-gated sodium channels alone, three mutations confer knock-down resistance to DDT and pyrethroids in *Anopheles gambiae s.l.* (Martinez-Torres et al. 1998, Ranson et al. 2000, Jones et al. 2012). Those mutations are L1014F, L1014S, and N1575Y. Insensitive acetylcholinesterase (ace1R) also confers resistance to both organophosphates and carbamates (Weill et al. 2004) and was reported to occur in *An. coluzzii* and *An. gambiae* by introgression (Djogbénou et al. 2008). The allele kdr *L1014F* was detected for the first time in West Africa (Martinez-Torres et al. 1998), and was termed kdr-west, whereas kdr *L1014S* was detected in East Africa and named kdr-east. The N1575Y mutation was reported in most West African countries and Central Africa (Jones et al. 2012). Those three mutations are reported in this study (especially at Mango, Efofami-Yéyé, and Nangbéto), and L1014F seems to be frequent at all localities, confirming it as a dominant resistance allele detected in *Anopheles* mosquitoes in Togo (Fig. 3), given excessive DDT use for malaria vector control in the early 1950s, and the later overuse of pyrethroids, especially in agricultural practices, in African countries. Because the Oti River crosses Mango, this locale cultivates rice with irrigation based in the river basin; which is a less strictly regulated

agricultural activity where farmers can use pesticides of all kinds for pest control. Efofami-Yéyé and Nangbéto belong to the Plateau Region of Togo, with a high human footprint in terms of agriculture, including ~165,000 agricultural households—more than in the other regions—according to the Ministry of Agriculture, Livestock, and Fisheries (DSAID 2013). In addition, this area is also known as a cotton-growing zone. These differences could explain the resistance pressure in *An. gambiae* and *An. coluzzii*. The East African kdr mutation was reported in West Africa first in Benin (Djègbe et al. 2011), and it has now spread to other West African countries. The *ace1* mutation is present in the neighboring countries (Dabiré et al. 2009, Djogbénou et al. 2011, Essandoh et al. 2013) at low frequencies, given the cost it represents for resistant individuals to survive and contribute to the next generation (Djogbénou et al. 2010). The spread of these mutations represents a serious threat to vector control efforts, and constitutes a worsening situation that needs urgent action to maintain malaria control (Ranson and Lissenden 2016).

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Supplementary Figure 1: Results of principal component analysis (PCA) applied to climatic variables.

Supplementary Figure 2: Geographical representation of principal component analysis (PCA) applied to climatic variables.

Supplementary Figure 3: Randomization test for carbamates insecticide resistance with a 90% threshold for presence of crops and percentage of tree cover.

Supplementary Table 1: Geographical coordinates for studied localities and median of insecticide mortality.

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