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Monitoring and molecular profiling of contemporary insecticide resistance status of malaria vectors in Guinea–Bissau

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ABSTRACT

Despite reduction in the prevalence of malaria, Guinea-Bissau (GB) is still widely affected by the disease that is primarily vectored by *Anopheles gambiae s.l.* mosquitoes. Monitoring mosquito susceptibility and investigating the insecticide resistance status is an integral part of malaria control actions. Here, mosquito populations from five regions of GB: Bafatá, Bissau, Buba, Cacheu and Gabu were monitored for species ID and insecticide resistance, using diagnostic and intensity WHO bioassays, as well as molecular assays. Phenotypic and molecular identification of species showed the presence of *An. gambiae s.s.* (S form), *An. coluzzii* (M form) and *An. arabiensis*, as well as rare *An. arabiensis/ An. gambiae* hybrids. Resistance to permethrin and deltamethrin was found in all *Anopheles* populations assayed, with the intensity of resistance for permethrin being moderate to high, as confirmed by bioassays performed at concentration intensities of 5X and 10X. Consistent to these findings, molecular analysis showed a higher frequency of knock-down resistance (kdr) mutations (L1014F, L1014S, reaching > 90% in some areas) compared to previous studies in the same region, as well as detected for the first time the presence of the super kdr mutation (N1575Y) in GB. The "iAche" (G119S) resistance mutation was also found in GB in low frequencies (up to 12.41%). Additionally, the synergistic PBO-permethrin bioassays suggested partial involvement of non target (metabolic and/or reduced penetration) resistance mechanism. Expression analysis of known pyrethroid metabolisers indicated the slight overexpression and possible association of the cytochrome P450s *CYP6Z1*, *CYP4G16* with the pyrethroid resistance phenotype. The findings should guide future evidence-based resistance management strategies in GB.

1. Introduction

Malaria is an infectious disease with a worldwide impact harming public health, economy and society in low- and middle-income regions (El-Houderi et al., 2019; Herekar et al., 2020). Five species of single-celled parasites of the genus *Plasmodium* can cause the disease, among which *P. falciparum* is the prevalent form (99.7%) in the African region in 2018, followed by *P. vivax* (75%). The infection is acquired by the bite of *Anopheles* female mosquitoes during blood-meal (Acharya et al., 2017).

Several countries in the African region are endemic to malaria, with populations at risk of contracting the disease annually. In 2018, 228 million cases and 405.000 fatalities related to malaria were estimated globally, 94% of the deaths in the WHO African region (WHO, 2018).

Guinea-Bissau (GB) is an African country widely affected by malaria. Although data indicate a marked reduction in its prevalence, the disease continues to be one of the major causes of morbidity and mortality remaining a major public health issue (WHO, 2018). The strategies of the National Malaria Control Program in GB are based on the early diagnosis by laboratory tests and early treatment with the recommended first-line medication, and effective vector control. This is performed using long-lasting impregnated nets (LLIN's) according to World Health Organization (WHO) guidelines (WHO, 2019).

Mass distribution campaigns of LLIN's throughout the country are carried out every three years, and were already performed in 2011, 2014, 2017. Assessments of LLIN's use have shown high coverage in

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many population groups. In 2017, 98% of the households in GB had at least one LLIN in use and LLIN's were generally used by 92% of the population. The next LLIN's campaign is planned for 2020 (MIS, 2017).

A major problem associated with the extensive use of insecticides targeting mosquitoes or agricultural pests is insecticide resistance. The widespread use of impregnated bed nets, such as LLIN's, may contribute to the selection of insecticide resistant mosquitoes, namely malaria vectors (WHO, 2019). Therefore, monitoring mosquito's insecticide resistance status was well-defined as an integral part of essential malaria control actions in GB (WHO, 2017, 2018). However, information on mosquito insecticide susceptibility in GB is scarce. Phenotypic susceptibility to insecticides was carried out in 2002 and 2009 (Dabiré et al., 2008; Konate, 2009). In 2002, *Anopheles gambiae* populations from four ecological regions of GB, namely, urban in Bissau, forest in Tombali, mangrove coastline in Cacheu and savanna in Gabu were tested against permethrin (0,75%) and DDT (4%) and were susceptible notwithstanding location or ecosystem. The highest relative frequency (0,14) of knock-down resistance (kdr) target site mutations was observed exclusively in the S molecular form (*An. colluzzii*) in the region of Gabu (Dabiré et al., 2008). In 2009, *An. gambiae* populations from the regions of Bissau and Gabu were studied, and overall susceptibility to permethrin and deltamethrin was observed. High frequency of kdr mutations in the Gabu region (0,218), and lower mutation rate in Bissau (0,129) were recorded, but further investigation was essential regarding the molecular analyses results (Konate, 2009). In 2017, a molecular taxonomic study of *Anopheles* spp. was performed in littoral of GB that belongs to the ''hybrid region'' for *An. coluzzii* and *An. gambiae* (Vicente et al., 2017). Insecticide resistance genes were also genotyped. High frequency of kdr mutations in *An. gambiae* populations (41%) and their absence in *An. coluzzii* was observed. However, in this study phenotypic susceptibility tests and metabolic resistance diagnosis were not performed.

Since LLIN's are widely distributed in GB every three years and considering the next LLIN's distribution is planned for 2020, it is of great importance to know the insecticide resistance status of the target mosquito vectors. Here, we tested pyrethroid insecticide resistance in *An. gambiae* populations from five study areas using WHO bioassays, with intensity insecticide doses and synergists. These populations were genotyped for species identification and target-site mutations. Detoxification genes that have been identified in *An. gambiae* metabolic resistance were also included in the analysis.

2. Materials and methods

2.1. Study area

Guinea-Bissau (GB) is a Western Africa country, bordering the North Atlantic Ocean, between Guinea and Senegal (12°N, 15°W). It comprises an area of 36,120 km^2 with 2026, 012 inhabitants. Four different ecosystems are identified, namely Savana-Guinean in the Gabu region, forest in Quinara region, the coast region of Cacheu, and an urbanized region in the Autonomous Section of Bissau, Bissau (Fig. 1, Suppl. Table S1).

These regions provide unique and well-characterized ecosystems and consequently a differential distribution of malaria vector populations. Therefore, sentinel stations for mosquito sampling were set in each one of them. Additionally, sampling was also conducted in Bafatá region, based on the commercial activity of this region and the presumed general use of insecticides.

2.2. Mosquito collections and sampling methodology

Fieldwork for the collection of immature forms of *Anopheles* spp. mosquitoes took place from 6 to 31 October 2018 in each of the GB sentinel regions. *Anopheles gambiae s.l.* larvae were collected at the end of the rain period from breeding sites in the regions of Buba, Gabu-Bafata, Bafata, Gabu and Bissau. The fieldwork was carried out for five continuous days at each location within each region and the breeding sites where mosquito immature collection was performed included rain puddles, rice paddies, marshes, tanks, drainage channels and pools. At each breeding site, all immature stages found were collected, from L1 to L4 larvae and pupae, registered and later transferred to the insectary of the National Laboratory of Public Health (LNSP), in Bissau. Reared adult mosquitoes were fed with sugar solution until the beginning of insecticide assays. The *An. gambiae s.s.* and *An. coluzzii* susceptible laboratory colonies, Kisumu and Ngusso, respectively, as well as the *An. arabiensis* susceptible laboratory colonies MOZ and Sekoru were also included in the study (Simma et al., 2019). Female adult mosquitoes were tested for resistance against pyrethroids and carbamates following the WHO bioassay protocol (WHO, 2016).

2.3. Insecticide susceptibility tests

Anopheles gambiae mosquitoes were tested with classic and intensity (5X and 10X) WHO insecticide susceptibility assays against the pyrethroids permethrin and deltamethrin, plus the synergist PBO assays following WHO recommendations (2016). Accordingly, five replicates were used for each insecticide test with pools of 25 to 30 mosquitoes three to five days old. All the mosquitoes used in the bioassays were individually stored in tubes with silica gel. For the species identification, target site and metabolic resistance analyses assays, the mosquitoes have been stored in RNA later in pools of ten.

2.4. Nucleic acid (NA) extraction from mosquito pools and individuals

Total DNA was extracted from individual mosquitoes using a magnetic beads-based approach with the Nuclisens Easymag (Biomérieux). In brief, sampled mosquitoes were individually grinded in Lysis Buffer (NUCLISENS® easyMAG, Biomérieux) using glass pearls in 2 ml eppendorfs. NA extraction was performed using the prepared lysate suspensions in the automated platform NUCLISENS® easyMAG (Biomérieux). Total NAs (both RNA and DNA) were also extracted from pools of mosquitoes (non-blood fed, 3–5 days old *An. gambiae s.l.* females, preserved in RNAlater) using a magnetic beads-based approach with the MagSi kit (MagnaMedics Diagnostics B.V.). The quantity and purity of DNA and total RNA were assessed spectrophotometrically via Nanodrop measurements. The quality of RNA was assessed by 1.0% w/v agarose gel electrophoresis.

2.5. Genotyping of mosquito samples and multiplex RT-QPCR for gene expression analysis

Identification of the *Anopheles gambiae s.l.* species complex and molecular forms were performed in individual mosquitoes by PCR-RFLP following the protocol described by (Scott et al., 1993) and (Fanello et al., 2002); screening for kdr mutations was performed according to (Bass et al., 2007). In mosquito pools, species identification and target site resistance mutation (L1014F, L1014S, N1575Y, G119S) determination were performed using the assays described (Bass et al., 2010 and Mavridis et al., 2018). Analysis of *An. gambiae* molecular forms (S, M) was based on the insertion polymorphisms of SINE200 retrotransposons within speciation islands (Santolamazza et al., 2008). When analysing mosquito pools the % allele frequency for the previously mentioned traits was calculated with regression models using a methodology that we have previously developed (Mavridis et al., 2018). The expression levels of eight well characterized genes which have been associated with insecticide resistance in *An. gambiae s.l.* (*CYP6P3, CYP6M2, CYP9K1, CYP6P4, CYP6Z1, GSTE2, CYP6P1, CYP4G16*) were determined as previously described (Mavridis et al., 2019). Reactions were performed in the Viia7 Real-Time PCR system (Applied Biosystems) using a one-step RT-PCR mastermix supplied by FTD (Fast-track diagnostics, Luxembourg) in a total reaction volume of

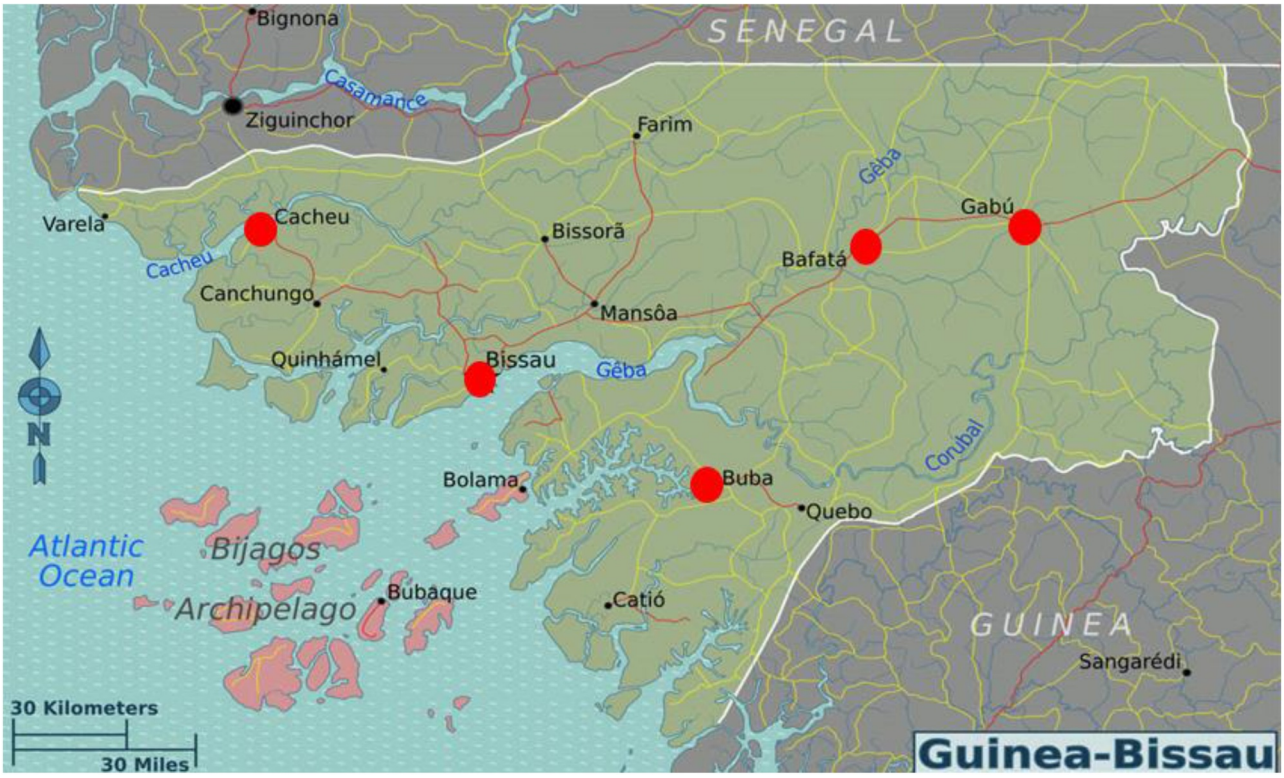

Fig. 1. Map of Guinea-Bissau showing the study's sites: Bissau, Cacheu, Gabu-Bafatá and Buba. Red dots indicate sampling regions.

10 uL. The thermal cycle parameters were: 50 ˚C for 15 min, 95 ˚C for 3 min, and 40 cycles of 95 ˚C for 3 s and 60 ˚C for 30 s, allowing a sample to result time of ~75 min. Samples were amplified in duplicates and each run always included a non-template control.

2.6. Statistical analysis

Calculation of fold-changes, 95% CIs and statistical significance was performed according to the Pfaffl method (Pfaffl, 2001). Graphs were constructed with the SigmaPlot software (v12.0).

3. Results

3.1. Insecticide susceptibility tests

A total of 2023 *Anopheles* mosquitoes, 90.2% females (N = 1824) and 9.8% males (N = 199), were used in the insecticide susceptibility bioassays. From these 78.2% (N = 1582) were exposed to the insecticides, 91.5% females (N = 1447) and 8.5% males (N = 135), and 21.8% (N = 441) were used as control, corresponding to 85.5% females (N = 377) and 14.5% males (N = 64) (Table 1).

Mortality rates for deltamethrin and permethrin were less than 98% in all regions, suggesting resistant populations to these pyrethroids. Tests to determine resistance intensity for permethrin at 5X and 10X concentrations also resulted in mean mortality rates of 47.0% and 86.0%, respectively and the synergistic PBO-permethrin bioassay performed for the SAB population resulted in a mortality rate of 57.7% (Table 1). These results indicate mosquito populations with moderate to high resistance intensity.

3.2. Molecular genotyping

3.2.1. Species ID

Mosquitoes from Buba, Gabu/ Bafata, Bafata and Gabu were identified as *An. gambiae s.s.* in their majority, with *An. coluzzii* mosquitoes ranging from 6.2% – 13.5%. In Bafata, Gabu and Gabu Bafata areas a very small proportion (1.3% – 4.0%) of *An. arabiensis* mosquitoes was also detected (Table 2, Suppl. Fig. S1).

A different species composition was observed in the Bissau population, where the 75.0% of mosquitoes were identified as *An. arabiensis* with the remaining belonging to *An. gambiae s.s.* (16.0%) and *An. coluzzii* (9.0%) species. We additionally genotyped individuals of the Bissau population and we found that 3.22% (2/62) are actually *An. gambiae/ An. arabiensis* hybrids.

3.2.2. Target site mutations (KDR L1014F/S, KDR N1575Y, iAche G119S)

The L1014F mutation (kdr-west) was found in very high frequencies (75.01 – 91.96%) across the Buba, Gabu/ Bafata, Bafata and Gabu populations. On the contrary, the L1014S (kdr-east) mutation was found in low frequencies (0.00 – 1.25%) across the Buba, Gabu/ Bafata, Bafata and Gabu populations (Table 3).

The Bissau population was characterized mainly by the presence of the L1014S allele (58.71%) and at lower (31.29%) frequency the presence of the L1014F allele. The combined kdr mutant allelic frequency reached 90.0% (Table 3). Individual mosquito genotyping from the Bissau region corroborated the high frequency of kdr mutations (79.4%) (Table 3).

The N1575Y mutation (super kdr) was detected in low frequencies across the study's populations. More specifically, the Gabu Bafata and Bafata populations showed the highest mutation frequency (17.56% and 17.47%, respectively), followed by Gabu (14.98%), Buba (12.98%) and Bissau (6.62%). We additionally genotyped individuals of the Bissau population and we found that 1575Y mutations were not detected in the *An. arabiensis* mosquitoes.

The iAche (G119S) mutation was either not detected (Bafata, Bissau) or detected in low frequencies across the study's populations; Buba showed a mutant allele frequency of 1.76%, in Gabu Bafata it reached 6.21% and Gabu showed the highest frequency across the study's populations (12.41%).

Table 1
Mortality rates in insecticide susceptibility bioassays.

Region	Insecticide	Test			Control		
		N	Replicates	M[% mortality]	N	Replicates	M[% mortality]
Bafatá	Deltamethrin 0.05%	25	1	33.3	30	1	10
	Permethrin 0.75%	105	4	45 ± 3.2	30	1	10
Buba	Deltamethrin 0.05%	103	4	21.2 ± 9.9	25	1	4
	Permethrin 0.75%	103	4	21 ± 8.3	29	1	0
	5X	115	4	45.8 ± 8.5	25	1	12
	10X	112	4	91.1 ± 1.9	30	1	0
Cacheu	Deltamethrin 0.05%	102	4	40.1 ± 15	47	2	10.7 ± 3.3
	Permethrin 0.75%	132	5	59.5 ± 22.9	54	2	5.6 ± 2.6
	5X	77	3	47.2 ± 19.5	24	1	4.2
Gabu	Deltamethrin 0.05%	107	4	12.1 ± 3.2	28	1	3.6
	Permethrin 0.75%	117	4	25.1 ± 12.1	25	1	12
	5X	102	4	39.1 ± 7.5	18	1	0
SAB	Deltamethrin 0.05%	105	4	44.8[a]	24	1	0
	Permethrin 0.75%	105	4	20.5 ± 7	25	1	0
	5X	105	4	56 ± 11.8	28	1	21.4
	10X	88	4	81 ± 11.9	30	1	0
	Permethrin	28	1	17.9	27	1	0
	PBO 4%	25	1	0	27	1	0
	PBO + Permethrin 0.75%	26	1	57.7	27	1	0

[a] Four replicates were performed, but by lapse, the mosquitoes were counted as total.

Table 2
Distribution of *An. gambiae s.s., An. coluzzii* and *An. arabiensis* species.

Population	Collection Date	% Species
Kisumu lab strain	N/A	100% *An. gambiae s.s.*
Ngusso lab strain	N/A	100% *An. coluzzii*
MOZ lab strain	N/A	100% *An. arabiensis*
Sekoru lab strain	N/A	100% *An. arabiensis*
Buba-1	Nov 2018	92.5% *An. gambiae s.s.* 7.5% *An. coluzzii*
Buba-2*	Oct 2018	76.6% *An. gambiae s.s.* 17.2% *An. coluzzii* 6.2% *An. arabiensis*
Gabu/Bafata	Nov 2018	82.9% *An. gambiae s.s.* 13.1% *An. coluzzii* 4.0% *An. arabiensis*
Bafata	Nov 2018	84.8% *An. gambiae s.s.* 13.5% *An. coluzzii* 1.7% *An. arabiensis*
Gabu-1	Nov 2018	92.5% *An. gambiae s.s.*6.2% *An. coluzzii*1.3% *An. arabiensis*
Gabu-2*	Oct 2018	80.8% *An. gambiae s.s.* 15.4% *An. coluzzii* 3.8% *An. arabiensis*
Bissau-1**	Nov 2018	16.0% *An. gambiae s.s.* 9.0% *An. coluzzii* 75.0% *An. arabiensis*
Bissau-2*	Oct 2018	14.1% *An. gambiae s.s.* 3.3% *An. coluzzii* 82.6% *An. arabiensis*

* Performed in individual mosquitoes; N/A: Not applicable.
** Genotyping of individuals from the Bissau-1 population revealed that 3.22% (2/62) were actually *An.gambiae/ An.arabiesis* hybrids.

3.2.3. Detoxification gene expression

We assessed the expression levels of seven detoxification genes (*CYP6P3, CYP6M2, CYP9K1, CYP6P4, CYP6Z1, CYP6P1, GSTE2*) that have been strongly associated with metabolic resistance (Chiu et al., 2008; Ibrahim et al., 2016; Müller et al., 2008; Ranson et al., 2000; Stevenson et al., 2011; Vontas et al., 2018) and one oxidative

Table 3
Incidence of resistance alleles in different populations of *An. gambiae s.l.* mosquitoes.

Population	Collection date	Sample size (alleles)	Mutant allele frequencies (mean ± SE) [range]			
			Pyrethroids		Carbamates / OPs	
			% kdr L1014F	% kdr L1014S	% kdr N1575Y	% iAChE
G119S						
Kisumu	N/A	60	0.00	0.00	0.00	0.00
Ngusso	N/A	60	0.00	0.00	0.00	0.00
MOZ	N/A	60	0.00	0.00	0.00	0.00
Sekoru	N/A	60	0.00	0.00	0.00	0.00
Buba	Nov.2018	100	84.12 ± 6.97 [64.24 - 100]	0.00	12.98 ± 4.42 [0.00 - 27.60]	1.76 ± 1.76 [0.00 - 8.78]
Gabu/Bafata	Nov.2018	100	80.44 ± 3.28 [68.57- 86.82]	1.00 ± 1.00 [0.00 - 5.0]	17.56 ± 4.33 [8.4 - 32.00]	6.21 ± 6.21 [0.00 - 31.07]
Bafata	Nov.2018	60	75.01 ± 12.6 [50.61- 92.43]	0.00	17.47 ± 1.11 [16.30 - 19.70]	0.00
Gabu	Nov.2018	80	91.96 ± 5.1 [78.71- 100]	1.25 ± 1.25 [0.00 - 5.0]	14.98 ± 3.77 [8.30 - 24.70]	12.41 ± 7.3 [0.00 - 27.92]
Bissau-1	Nov.2018	200	31.29 ± 4.84 [19.00 - 55.00]	58.71 ± 7.2 [27.50–81.00]	6.62 ± 2.46 [0.00 - 20.30]	0.00
Bissau-2*	Oct. 2018	102	79.4%	N/A	N/A	

* performed in individual mosquitoes; N/A: Not applicable.

Table 4
Detoxification gene expression analysis in the An. gambiae s.s./An. coluzzii resistant mosquito populations compared to the susceptible laboratory strains.

Population	Comparison	Detoxification gene fold changes (95% CI)							
		CYP6P3	*CYP6M2*	*CYP9K1*	*CYP6P4*	*CYP6Z1*	*GSTE2*	*CYP6P1*	*CYP4G16*
Buba	Vs KIS	0.22 (0.14–0.39)	0.13 (0.060–0.22)	0.832 (0.48–1.81)	0.41 (0.25–0.57)	1.18 (0.64–2.35)	0.84 (0.52–1.22)	0.90 (0.64–1.17)	1.07 (0.78–1.79)
	Vs NG	0.083 (0.058–0.14)	0.095 (0.048–0.15)	0.26 (0.19–0.34)	0.34 (0.26–0.41)	0.71 (0.47–1.06)	0.24 (0.16- 0.33)	0.43 (0.33–0.49)	0.65 (0.52–0.85)
Gabu/Bafata	Vs KIS	0.192 (0.12–0.27)	0.239 (0.19–0.34)	2.44 (0.94–6.34)	0.76 (0.48–1.07)	1.95 (0.73–5.23)	1.33 (0.97–1.87)	1.03 (0.82–1.37)	2.11 (1.47–3.64)
	Vs NG	0.071 (0.048–0.098)	0.18 (0.15–0.23)	0.77 (0.68–0.88)	0.63 (0.52–0.78)	1.17 (0.74–1.85)	0.38 (0.12–1.18)	0.49 (0.44–0.59)	1.29 (0.97–1.73)
Bafata	Vs KIS	0.45 (0.21–0.84)	0.23 (0.036–0.89)	1.13 (0.44–4.27)	0.92 (0.41–1.50)	1.84 (0.96–3.55)	0.69 (0.37–1.16)	1.02 (0.62–1.55)	1.64 (1.10–3.07)
	Vs NG	0.17 (0.086–0.29)	0.17 (0.019–1.43)	0.36 (0.092–1.36)	0.77 (0.41–1.09)	1.10(0.83–1.49)	0.20 (0.11–0.31)	0.49 (0.16–1.46)	1.00 (0.73–1.48)
Gabu	Vs KIS	0.65 (0.30–1.11)	0.32 (0.094–1.05)	2.71 (0.84–8.72)	0.67 (0.45–0.89)	2.19* (1.41–4.08)	1.35 (1.04–1.64)	0.83 (0.63–1.16)	1.46 (1.12–2.32)
	vsNG	0.24 (0.12–0.40)	0.23 (0.20–0.30)	0.85 (0.50–1.46)	0.56 (0.49–0.65)	1.31*(1.05–1.62)	0.39 (0.13–1.19)	0.39 (0.33–0.50)	0.89 (0.74–1.10)

KIS: Kisumu susceptible strain (*An. gambiae s.s*); NG: Ngusso susceptible strain (*An. coluzzii*); BU: Buba; GB: Gabu/Bafata; BA: Bafata; GA: Gabu Bold letters indicate statistically significant upregulation, asterisks (*) indicate consistent upregulation compared to both susceptible strains.

decarboxylase (*CYP4G16*) that catalyses epicuticular hydrocarbon biosynthesis and is implicated in cuticle resistance in *An. gambiae* (Balabanidou et al., 2016). No striking detoxification gene overexpression was detected in the study's populations.

A statistically significant, but rather mild, overexpression of *CYP6Z1* was detected in the Gabu *An. gambiae s.s./ An. coluzzii* population. More precisely, *CYP6Z1* was found to be overexpressed 2.19 folds (95% CI = 1.41–4.08) compared to the Kisumu susceptible strain. The statistical significance of this overexpression was also corroborated with the Ngusso comparison (1.31 folds, 95% CI = 1.05–1.62). No other insecticide resistance related gene from the ones studied was found to be differentially expressed in the *An. gambiae s.s./ An. coluzzii* resistant populations compared to the susceptible lab strains (Table 4 and Suppl. Fig. S3D).

The Bissau *An. arabiensis* population also showed an increase of *CYP6Z1* expression levels by 23.8 folds (95% CI = 14.7–40.1) compared to the MOZ, and 1.71 folds (95% CI = 1.08–2.59) compared to the Sekoru *An. arabiensis* susceptible strains. *CYP4G16* was additionally upregulated in the later *An. arabiensis* population by 11.5 folds compared to MOZ (95% CI = 6.05–28.3), and 1.81 folds (95% CI = 1.23–2.53) and Sekoru susceptible strains (Table 5, Suppl. Fig. S4).

4. Discussion

Recent studies on phenotypic susceptibility to insecticides using the WHO bioassays tests have been showing the presence of *An. gambiae* resistant populations to pyrethroids in Sub-Saharan African countries. In Kenya's Kwale County, mortality rates of 75.48% and 78.11% for deltamethrin and permethrin, respectively, were observed, which clearly confirm the presence of resistance (Kiuru et al., 2018). In Nigeria, resistant populations of *An. gambiae* to lambdacyhalothrin, permethrin, deltamethrin, and to DDT were reported (Oyewole et al., 2018). Populations of *An. arabiensis* from Dakar, Senegal, showed resistance to pyrethroids and organochlorides insecticides, and were only susceptible to organophosphates (Dia et al., 2018). These studies clearly indicate the global trend for insecticides resistance of the main malaria vectors in Africa and highlight the importance of analysing their susceptibility status as an integral part of the essential actions to fight malaria, since the presence of resistant populations would compromise the efficiency of LLIN's.

In this study priority was given to the most widely used insecticides in GB and the ones recommended by WHO for use in LLIN's, namely pyrethroids. The observed association between the widespread use of LLIN's and the evolution of resistance to insecticides raises concern about their continued effectiveness in preventing malaria.

The *An. gambiae* populations tested in this study were resistant to pyrethroids. Phenotypic resistance and pyrethroid resistance genes were for the first time identified in 2008 and 2009 in the regions of Bissau and Gabu (Dabiré et al., 2008) and our results were aligned with those, confirming a continuous and strong evolution of resistance most probably due to increased use of LLIN's and other control methods using the same class of insecticides (e.g. agriculture, gardening).

Regarding molecular mechanisms of resistance to pyrethroids in GB, our study documents that kdr mutations are found in very high frequencies close to fixation across the study's sites. A previous study conducted in 2008 reported low frequencies (2.00 – 14.00%) for the kdr L1014F mutation and only in two sites: Bissau and Gabu (Dabiré et al., 2008). Consequently, this study demonstrates a striking escalation of mutant kdr allele frequency. The 1014F and/or 1014S alleles have been previously detected, in some cases in high frequencies, in neighbouring countries, including Senegal (Ndiath et al., 2015), Guinea (Stica et al., 2019) and Mali (Cisse et al., 2015). The (super kdr) N1575Y mutation was detected here for the first time in GB, albeit in low frequencies (6.62–17.56%) across the study's populations. The presence of N1575Y in neighbouring *An. gambiae* populations from Mali (Mavridis et al.,

Table 5
Detoxification gene expression analysis in the *An. arabiensis* resistant mosquito population compared to the susceptible laboratory strains.

Population	Comparison	Detoxification gene fold changes (95% CI)							
		CYP6P3	*CYP6M2*	*CYP9K1*	*CYP6P4*	*CYP6Z1*	*GSTE2*	*CYP6P1*	*CYP4G16*
Bissau	vs SEK	0.049 (0.021–0.13)	1.06 (0.45–3.12)	0.91 (0.47–1.47)	0.62 (0.25–1.37)	1.71* (1.08–2.59)	0.15(0.084–0.27)	0.46 (0.34–0.69)	1.81* (1.23–2.53)
	vs MOZ	0.289 (0.11–0.78)	4.65 (0.77–28.1)	3.62 (2.07–5.11)	3.89 (1.56–8.04)	23.8*(14.7–40.1)	1.50 (0.89–2.46)	0.78 (0.35–1.37)	11.5* (6.05–28.3)

SEK: Sekoru susceptible strain (*An. arabiensis*); MOZ: MOZ susceptible strain (*An. arabiensis*) Bold letters indicate statistically significant upregulation, asterisks (*) indicate consistent upregulation compared to both susceptible strains.

2018), and Guinea (Stica et al., 2019) indicates the introduction of this mutation in the overall area. Interestingly, during our molecular species identification, we were also able to detect *An. arabiensis/ An. gambiae* hybrids. Such hybrids are very rare (Weetman et al., 2014) and could represent generations beyond F1 (backcrosses). They could readily lead to adaptive introgression of genetic variants relevant for vector control (Weetman et al., 2014), such as the super kdr (N1575Y) allele that has not been detected up to date in *An. arabiensis* mosquitoes.

The synergistic PBO-permethrin bioassay applied to the population of *Anopheles* SAB, with mortality below 98%, but higher than in the sample exposed only to permethrin suggests partial involvement of non target (metabolic and/or reduced penetration-based) resistance mechanism. Gene expression analysis indicated a, rather mild, upregulation of *CYP6Z1,* which was observed both in the *An. gambiae s.s. / An. coluzzii* and the *An. arabiensis* populations. *CYP6Z1* has been previously shown to be capable of metabolizing DDT (Chiu et al., 2008) and to be frequently over transcribed in pyrethroid and DDT resistant *An. arabiensis* populations (Nardini et al., 2013) and *An. gambiae* populations (Nikou et al., 2003; Vontas et al., 2018; Main et al., 2018). *CYP6Z1* has been previously found to be overexpressed in resistant *An. coluzzii* individuals from Mali following sublethal permethrin exposure (Main BJ, et al. 2018). Interestingly, *CYP4G16*, a gene which catalyses epicuticular hydrocarbon biosynthesis, and is implicated in cuticle resistance, *i.e.* epicuticular thickening, due to elevated cuticular hydrocarbons (CHCs) that leads to lowered insecticide uptake, was found to be upregulated in both *An. gambiae* and *An. arabiensis* populations of the study (Balabanidou et al., 2016). Increased transcription of *CYP4G16,* has been previously detected in pyrethroid-resistant *An. arabiensis* (Matowo et al., 2014) and *An. gambiae* (Yahouédo et al., 2017) mosquitoes. This finding suggests an, at least partial, contribution of cuticle resistance to the phenotypes of the present study's populations, yet this needs to be supported by further lines of evidence in a future work. Our study is the first to analyze insecticide resistance related gene expression profiles in Guinea-Bissau. Although no phenotypic data were available regarding organophosphate and carbamate resistance for the study's populations, we were able to detect the iAche (G119S) mutation in low frequencies (0.00 – 12.41%). The G119S mutation has been previously detected in Guinea (Stica et al., 2019) and Mali (Cisse et al., 2015), where it was associated with bendiocarb and/or fenitrothion resistance. The first detection of “iAche” in Guinea-Bissau we report herein is alarming and would be interesting to investigate the possible presence and the extend of phenotypic resistance to carbamates/organophosphates in a future study.

5. Conclusion

In conclusion, considerable levels of resistance to permethrin and deltamethrin was found in all *Anopheles* populations assayed from GB. Molecular analysis showed a high frequency of kdr, as well as detected the super kdr mutation (N1575Y). The synergistic PBO-permethrin bioassays indicating partial involvement of non-target (metabolic and/or reduced penetration) resistance mechanism, suggesting that the use of PBO combination would improve the efficiency of this control application. More work is required, in order to determine the exact operational impact of the resistance status recorded, in malaria incidence in GB.

CRediT authorship contribution statement

Ronise Silva: Methodology, Investigation, Resources, Writing - original draft. **Konstantinos Mavridis:** Investigation, Writing - original draft, Visualization, Formal analysis. **John Vontas:** Investigation, Methodology, Writing - review & editing, Validation. **Amabélia Rodrigues:** Conceptualization, Funding acquisition, Project administration. **Hugo Costa Osório:** Methodology, Validation, Writing - review & editing, Supervision.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105440.

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