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Influence of physico-chemical and microbiological parameters of mosquito larval habitats on the species composition and insecticide resistance of *Anopheles gambiae sensu lacto* in the cities of Bohicon and Parakou in Benin

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ABSTRACT

Despite successful of insecticide-treated nets and indoor residual spraying, malaria continues to be a major threat in Benin due to insecticide resistance in its vectors. This study aimed to address knowledge gaps regarding the impact of environmental factors on insecticide resistance in *Anopheles gambiae* populations in urban areas. Larval surveys in Bohicon and Parakou assessed physicochemical properties and bacterial loads, followed by WHO susceptibility assays on emerging adult mosquitoes. Anopheles species were identified through PCR and *Kdr L1014F/S*, *N1575Y* and *G119S* mutations were investigated using TaqMan assays. Results showed high resistance of Anopheles mosquitoes to pyrethroid insecticides (mortality rate: 16.9% to 87.27%). Additionally, molecular analysis revealed a predominance of *A. coluzzii* in Bohicon (60.7%) and *A. gambiae* in Parakou (8.1%), with high frequencies of the *kdr L1014F* (56% to 88%). Furthermore, physicochemical and microbiological analysis correlated significantly with *A. gambiae*, particularly regarding pH, turbidity, and fecal coliforms. Moreover, dissolved oxygen positively associated with both *A. coluzzii* and *A. gambiae* presence. *Kdr L1014F* allele showed significant correlations with temperature ($r = 0.906$; $P = 0.039$) in Bohicon and salinity ($r = -0.924$; $P = 0.022$) in Parakou, underscoring environmental influences on resistance patterns. The study underlined the importance of contextual approaches in in urban vector control efforts.

Keywords: *Anopheles gambiae*, Environmental Factors, Species, Insecticide Resistance, Urban Areas, Benin.

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INTRODUCTION

Malaria is a serious mosquito-borne parasitic disease which is responsible for millions of deaths in tropical and subtropical countries (Wangrawa et al., 2015). To control the malaria, the implementation of Roll Back Malaria initiatives has led to significant achievements through the widespread use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS), resulting in decreased of mortality and morbidity rates in malaria-endemic regions (Sougoufara et al., 2020). However, for a few decades there has been a growing emphasis on addressing the impact of malaria in urban settings of these endemic regions (Mathanga et al., 2016). Indeed, the urban areas, particularly in sub-Saharan African, represent dynamic environments where various factors, including rapid growth and density of population, rapid infrastructure development, and diverse human activities, intricately influence the transmission dynamics of malaria (Mathanga et al., 2016). Despite progress in public health efforts, malaria remains a major problem due to challenges in managing the environment and controlling mosquitoes, especially with the rise of insecticide resistance (Wangrawa et al., 2015).

The development of insecticide resistance in malaria vectors is attributed to the recurrent use of insecticides in vector control and agriculture, as well as exposure to anthropogenic or natural xenobiotics in mosquito breeding sites (Demissew et al., 2022). Several studies emphasized the significant roles played by environmental factors, such as climate and physicochemical properties, in shaping mosquito biology, influencing their distribution and abundance across diverse ecological areas (Muturi et al., 2008). Of these physicochemical properties, dissolved oxygen, pH, turbidity, total dissolved solids, water hardness and the presence of various ions such as chloride and nitrate, were known to influence the presence and abundance of larval mosquito species, including *Anopheles gambiae* (Seal et al., 2023). Despite this understanding, limited knowledge exists regarding the influence of these environmental factors on the resistance/susceptibility status and resistance mechanisms in Anopheles populations from Africa.

In Nigeria, recent studies have showed potential associations between molecular mutations, detoxification enzyme activity, and physicochemical factors in pyrethroid resistance (Ononamadu et al., 2020). Moreover, Kabula et al. (2011) revealed that there are correlations between specific chemical elements and the occurrence of resistance to insecticides. This interaction interplayed between genetic, ecological, and environmental factors underscores the complexity of insecticide resistance within mosquito populations. Consequently, it is urgent to understand the specific determinants driving insecticide resistance in order to conceive effective vector control strategies. This study aimed to assess the environmental factors influencing the species distribution and insecticide resistance in *Anopheles gambiae* populations in urban settings of Bohicon and Parakou in Benin.

MATERIALS AND METHODS

Study sites

The present study was conducted from August to September 2020, and January to August 2021 in the cities of Bohicon and Parakou, located in south and north-eastern Benin, respectively (Figure 1).

Bohicon (7°10'41.7"N, 2°4'0.1"E) features a transitional subequatorial climate, with two distinct rainy seasons occurring from April to June and from September to November. This city covers an area of 44 square kilometers and is home to a population of 171,781 inhabitants. The average annual rainfall is approximately 1,025 mm, and the temperature ranges from 25 to 34°C. The primary livelihoods of the population in Bohicon revolve around commerce, craftsmanship, as well as urban and peri-urban agriculture (Houngnihin, 2006).

Parakou (9°20'13.8' N, 2° 37'49.1"E), is situated at an average altitude of 350 meters above sea level and spans an area of 441 square kilometers. It is inhabited by a population of 254,254 individuals. Parakou is characterized by a South-Sudanese climate, which is tropical and humid, with a single rainy season occurring from May to October. The city receives an average annual rainfall of 1,200 mm, with the highest precipitation typically observed between July and September. The temperature ranges from 22 to 34°C with the lowest temperatures recorded in December and January. Major economic activities in Parakou include manufacturing, commerce, and urban agriculture (Kora, 2006).

Mosquito larval collection and mosquito rearing

A. gambiae s.l larvae were collected during the rainy season (from August to September) and dry season (January). The sampling method consisted in exploring all potential anopheles breeding sites by using the standard dipping method (Silver and Service, 2007). The presence/absence of mosquito larvae was determined after conducting 25 dips in breeding site. If larvae were found, they were carefully transferred to plastic containers. The number of both larvae and pupae was recorded, and the larval density was calculated as the ratio of the number of larvae collected per dip, following the methodology described by Service and Silver (2007).

Larvae and pupae collected were transported to the insectary of the laboratory of “Ecole Normale Supérieure de Natitingou” for rearing. The insectary conditions were: temperature 25°C to 33°C and humidity 70% to 80% with a 12-hours day/night cycles. After emergence, mosquitoes were fed with cotton wool pads soaked with 10% sucrose solution.

Physi-cochemical and bacterial analysis of mosquito larval habitats

The water's properties at each sampling site were assessed and recorded. This assessment included measuring pH with a Hanna HI 991001 pH meter, turbidity using a Hanna HI 93703 Turbidity Meter, dissolved oxygen (DO) with a WTW OXI 3205 oximeter, conductivity, total dissolved solids (TDS), salinity, and temperature using the VWR CO300 multiparametric conductivity meter.

Following the measurements, a minimum of 200 milliliters of water was collected from each sampling site and placed into a sterile conical flask. Subsequently, the flask was stored in an icebox and transported back to the laboratory for the analysis of bacterial pollution in the larval habitats.

Microbiological pollution in the different breeding sites was assessed through the isolation and identification of total coliforms and *Escherichia coli*, as well as fecal coliforms according to the technique of filtration through a nitrocellulose membrane of 0.45 µm on Chromogenic Coliform Agar, following the procedures detailed in the protocol of Nonfodji et al. (2020)

Insecticide susceptibility tests

Protocols and standard insecticide treated papers supplied by WHO were used to test susceptibility of *A. gambiae* mosquitoes from different sites to various insecticides. Insecticides belonged to the three major public health classes: permethrin 0.75% (pyrethroids type I), deltamethrin 0.05% (pyrethroids type II), pirimiphos methyl 0.25% (organophosphate) and bendiocarb 0.1% (carbamate). For each insecticide, approximately 20 non-blood-fed female mosquitoes aged from 3 to 5 days, were introduced into each test tube. Six test tubes were used: two control and four treated papers to expose mosquitoes. In the control tubes, impregnated papers with insecticide diluent only (i.e., acetone) were used, whereas treated papers were impregnated with diagnostic doses of insecticide. After 60 min of exposure, the mosquitoes were transferred to observation tubes containing untreated paper, with free access to 10% honey solution. Mortalities were recorded after 24 hours and the susceptibility status of the population was ranged according to the WHO criteria (WHO, 2016). Dead and alive mosquitoes from bioassay were separately kept in Eppendorf tubes at -20°C for further molecular analyses.

Molecular identification of *Anopheles* species

In each city, the samples of *A. gambiae* female from bioessays were analysed at the molecular level. So, genomic DNA of both alive and dead mosquitoes was extracted using protocol of Livak (1984). Specific DNA sequences were amplified using SINE-PCR

(Santolamazza et al., 2008) to identify species of *A. gambiae* s.l. Morover, TaqMan assays with two labelled fluorochromes probes FAM and HEX/VIC were used to detect the presence *of kdr L1014S* and *kdr L1014F* mutations, or the presence of *N1575Y* and *G119S* mutations.

Data analysis

The World Health Organization criteria were used to determine resistance status of mosquito population as follows: Mortality rate > 98%: susceptible mosquito population; Mortality rates ranged between 90 – 98%: suspected resistance in the mosquito population; Mortality rates < 90% : resistant mosquito population to the insecticide (WHO, 2016). Mortality rates of *A. gambiae* populations were compared using Fisher's exact test.

Genotype distributions were recorded in an Excel datasheet and analysis performed using SPSS 25.0. Allelic frequencies were calculated using the following formula $f(R) = (2n.RR + n.RS)/2N$, where n is the number of mosquitoes of a given genotype, RR represents the homozygote resistance allele, RS represents the heterozygote resistance allele, SS the susceptible allele, and N is the total number of mosquitoes tested.

Chi-square test (X^2) was used to compare insecticide resistance profiles in different breeding sites. The relationship between water properties, distribution of *A. gambiae* species, insecticide resistance profiles and kdr allele was determined using Pearson bivariate correlation analysis and Welch's test. All levels of statistical significance were determined at P < 0.05.

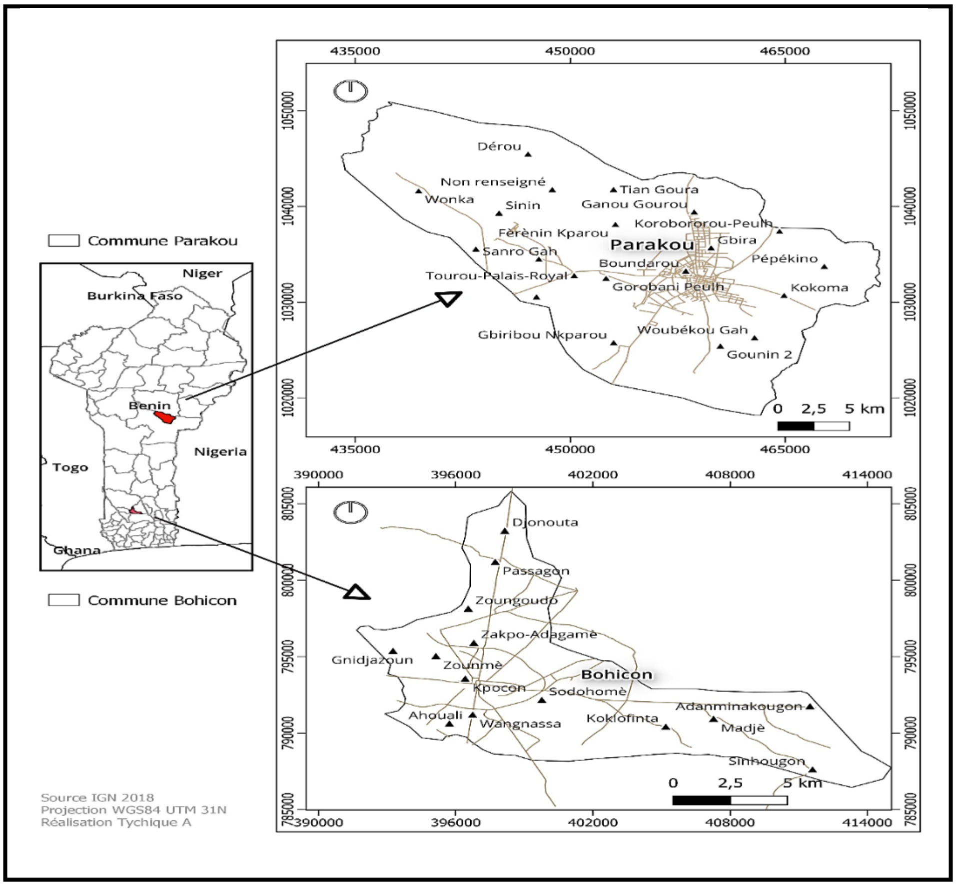

Figure 1: Map of the study sites.

RESULTS

Typology of *Anopheles* larval habitats

A total of 172 breeding sites were encountered, with 81 identified in Bohicon and 91 in Parakou during the collection periods (Table 1). Notably, 154 larval habitats were recorded during the rainy season, while in the dry season, it was 18. Moreover, 13 types of mosquito breeding sites were identified in study sites notably gutters, tires, vegetable farms, puddles, tire tracks, swamps, pits, water containers, hoof imprints, cans, ponds, under bridge. The majority of breeding sites was identified as temporary (89.5% ; P < 0,0001), irrespective of the season. Indeed, during the rainy season, a substantial frequency of temporary breeding sites was observed, accounting for 95% in Bohicon and 85% in Parakou. No difference was observed when we compared these characteristics between urban and non-urban areas within the respective study cities (P > 0.05).

In Bohicon, significant proportions of larval habitats were found in urban areas compared to non-urban environments (χ2 = 10.220 ; df = 1 ; P = 0.0014). However, in Parakou, no difference was observed between the proportions of larval habitats found in urban and non-urban areas (χ2 = 1.801 ; df = 1 ; P = 0.1796).

Physico-chemical and microbiological characteristics of *A. gambiae* s.l. larval habitats

The physico-chemical and microbiological characteristics of anopheline breeding sites in Bohicon and Parakou were presented in Tables 3 and 4. The analysis revealed differences between both cities (p= 0.0171). In Bohicon, lower temperature (30.41 ± 3.51°C) was observed, compared to Parakou (32.31 ± 3.73°C). In terms of pH, there is no significant difference between Bohicon (7.81 ± 0.77) and Parakou (7.90 ± 1.14). Moreover in Bohicon, total dissolved solids (TDS), conductivity and salinity were significantly lower than those observed in Parakou, (P= 0.0001). On the other hand, turbidity in Bohicon (247.39 ± 279.37 NTU) is significantly higher (P = 0.0045) than the one observed in Parakou (99.56 ± 176.41 NTU). No significant difference is observed in dissolved oxygen levels (P= 0.9741) between Bohicon (2.47 ± 1.35 mg/L) and Parakou (2.46 ± 1.50 mg/L).

Concerning microbiological characteristics, there was no difference in total coliforms (P = 0.0852) and fecal coliforms concentrations (P = 0.0934) between Bohicon (total coliforms: 95307.69 ± 82629.07 UFC/100 mL ; fecal coliforms : 738.46 ± 1114.70 UFC/100 mL) and Parakou (total coliforms : 1560667.64 ± 5580824.768 ; fecal coliforms : 93068.47 ± 360990,31). However, the concentration of *Eschericha coli* was significantly higher (P= 0.0486) in Parakou (369262.94 ± 1207464.26 UFC/100 mL) compared to Bohicon (5000 ± 6720.61 UFC/100 mL) (Table 2).

In Parakou, temperature (P = 0.0081), conductivity (P = 0.0013), salinity (P = 0.0195) and TDS (P = 0.0001) were significantly higher in urban sites than non-urban sites. However, dissolved oxygen (P = 0.0003) and turbidity (P = 0.0001) were significantly higher in non-urban areas.

Insecticide resistance status of *A. gambiae* s.l populations from study cities

The results of bioassays showed a high resistance of *A. gambiae* mosquitoes to permethrin with mortality rate of 44.84% in Bohicon (Figure 2). The tested mosquitoes developed resistance to deltamethrin across both study cities. In Bohicon, the mortality rate ranged from 25.95% to 87.27%, while in Parakou, it varied from 16.94% to 83.07%. Within the same city, mortality rates to deltamethrin varied from one district to another. In Parakou, a significant difference in the resistance level to deltamethrin (P < 0.0001) was observed between urban and non-urban areas. However, in Bohicon, no significant difference was observed between these areas (P = 0.0219) (Table 4). Conversely, in both Parakou and Bohicon, the tested females of *A. gambiae* s.l from the different breeding sites were all susceptible to bendiocarb (carbamates) and pirimiphos-methyl (organophosphates).

Species composition

A total of 521 specimens of *A. gambiae* s.l. collected from various breeding sites in Bohicon and Parakou were identified by PCR technique. In both cities, *A. coluzzii* and *A. gambiae* were found in sympatry but in different proportions. In Bohicon, the anopheline population was composed of *A. coluzzii* (n= 145 ; 60.7%) and *A. gambiae* (n= 94 ; 39.3%). But, in Parakou, it was *A. gambiae* which was predominant species (n= 259 ; 91.9%) in relation to *A. coluzzii* (n= 23 ; 8.1%) (P <0.0001). Furthermore, in Bohicon and Parakou, all identified *A. coluzzii* mosquitoes were exclusively found in urban areas (Table 5). As for the distribution of *A. gambiae,* all mosquitoes identified in Bohicon were found in urban areas, while no difference was noted in their distribution within urban and non-urban areas of Parakou (P = 0.2422) (Table 5).

Correlation between breeding sites properties and species composition

The analysis of association between physicochemical parameters and bacterial loads with species of *A. gambiae* complex showed that pH (r = - 0.840; P < 0.0001), turbidity (r = - 0.74; P < 0.0001), and fecal coliform load (r = - 0.52; P = 0.0019) negatively influenced the presence of *A. gambiae*. The presence of *A. coluzzii* was significantly and negatively impacted by pH (r = - 0.63; P = 0.024), conductivity (r = - 0.53; P = 0.037), and TDS (r = - 0.59; P = 0.0003) (Table 6). Conversely, dissolved oxygen is the only parameter positively and significantly associated with the presence of *A. coluzzii* and *A. gambiae* in different mosquito larval habitats (Table 5).

A. gambiae s.l. resistance gene

All the 521 *A. gambiae* s.l. identified was subjected to the PCR for searching the presence of different mutations. The results showed that *kdr L1014F* mutation was found in both *A. gambiae* (0.85) and *A. coluzzii* (0.83) at similar allelic frequencies (P > 0.05). Also its allelic frequency was high in the two species from Bohicon (0.84) and Parakou (0.72) (Table 7). In Parakou, the variation in the frequency of the *kdr L1014F* mutation between these two species was significant (P < 0.0001). This kdr allelic frequency was positively correlated with bioassays data. No significant difference was observed between the frequencies of the kdr L1014F mutation within these two cities (P = 0.2098).

The *Kdr L1014S* and *N1575Y* mutations were found exclusively in the populations of *A. gambiae* with respective average frequencies of 0.007 and 0.03 at Bohicon and Parakou.

Physico-chemical parameters of larval habitats and distribution of Kdr L1014F allele

The analysis of association between breeding sites parameters and *Kdr L1014F* allele revealed that salinity was the only physico-chemical parameter significantly correlated with *Anopheles* resistance to deltamethrin (r = -0.645; P < 0.05). Furthermore, the analysis of relationship between the frequency of *kdr L1014F* alleles (both homozygous and heterozygous) and the physico-chemical characteristics of larval habitats showed significant correlation. Specifically, there was a significant association between the allelic frequencies of Kdr L1014F with temperature (r = 0.906; P = 0.039) in Bohicon, and salinity (r = -0.924; P = 0.022) in Parakou (Table 8).

Table 1: Characteristics of breeding sites sampled in Bohicon and Parakou in Benin.

				Duration of water		**Origin**	
Seasons	**Cities**	**Types of sites**	**Total of breeedig sites**	**Temporary n (%)**	**Permanent n (%)**	**Natural n (%)**	**Artificial n (%)**
Rainy season	Bohicon	Non-urban areas	23	23 (100%)	-	18 (78.3%)	5 (21.7%)
		Urban areas	50	46 (92%)	4 (8%)	36 (72%)	14 (28%)
	Parakou	Non-urban areas	45	41 (91.1%)	4 (8.9%)	17 (37.8%)	28 (62.2%)
		Urban areas	36	28 (77.8%)	8 (22.2%)	17 (47.2%)	19 (52.8%)
Dry season	Bohicon	Non-urban areas	2	2 (100%)	-	-	2 (100%)
		Urban areas	6	6 (100%)	-	2 (33.3%)	4 (66.7%)
	Parakou	Non-urban areas	7	5 (71.4%)	2 (28.6%)	4 (57.1%)	3 (42.9%)
		Urban areas	3	3 (100%)	-	-	3 (100%)
Total			172	154 (89.5%)	18 (10.5%)	94 (54.7%)	78 (45.3%)

Table 2: Physico-chemical and bacterial characteristics of breeding sites in the cities of Bohicon and Parakou.

	Temperature (ºC)	**pH**	**Turbidity (NTU)**	**Conductivity (µS/cm)**	**Dissolved oxygen (mg/L)**	**Salinity (g/L)**	**TDS (mg/L)**	**Total coliforms (UFC/100mL)**	**Fecal coliforms (UFC/100mL)**	***E. coli* (UFC/100mL)**
Bohicon	30.41 ± 3.51	7.81 ± 0.77	247.39 ± 279.37	308.71 ± 241.42	2.47 ± 1.35	0.15 ± 0.13	171.29 ± 135.66	95307.69 ± 82629.07	738.46 ± 1114.70	5000 ± 6720.61
Parakou	32.31 ± 3.73	7.90 ± 1.14	99.56 ± 176.41	615.88 ± 366.0	2.46 ± 1.50	0.31 ± 0.20	440.22 ± 268.02	1560667.64 ± 5580824.768	93068.47 ± 360990,31	369262.94 ± 1207464.26
P-value	0.0171	0.6677	0.0045	< 0.0001	0.9741	< 0.0001	< 0.0001	0.0852	0.0934	0.0486

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Table 3: Physico-chemical and bacterial characteristics of breeding sites from urban and non urban areas in Bohicon and Parakou.

Parameters	**Bohicon**			**Parakou**		
	Non-urban breeding sites	**Urban breeding sites**	**P value**	**Non-urban breeding sites**	**Urban breeding sites**	**P value**
Temperature (°C)	31.77 ± 3.26	29.76 ± 3.49	0.0669	28.75 ± 2.69	31.32 ± 3.26	0.0081
pH	8.02 ± 0.81	7.72 ± 0.74	0.2182	7.62 ± 0.47	7.65 ± 0.58	0.8544
Turbidity (NTU)	172.17 ± 210.35	283,71 ± 303,86	0.2012	247.66 ± 192.85	34.25 ± 40.88	0.0001
Conductivity (µS/cm)	245.41 ± 97.00	341.62 ± 286,09	0.2014	287.42 ± 196.53	515.17 ± 226.4	0.0013
Dissolved oxygen (mg/L)	2.39 ± 0.91	2.52 ± 1.53	0.7587	3.68 ± 1.37	2.12 ± 1.04	0.0003
Salinity (g/L)	0.14 ± 0.14	0.156 ± 0.13	0.7044	0.13 ± 0.10	0.22 ± 0.14	0.0195
TDS (mg/L)	134.88 ± 55.86	190.23 ± 160.38	0.1909	179.97 ± 116.09	384.62 ± 184.5	0.0001
Total coliforms (UFC/100mL)	114000 ± 54848.88	79285.71 ± 102465.79	0.2174	308333.33 ± 170800.32	2817308,33 ± 9191894,27	0.1775
Fecal coliforms (UFC/100mL)	650 ± 1098.63	814.28 ± 1210.27	0.6569	23716.67 ± 29480.67	207378,83 ± 600132,64	0.1323
E. Coli (UFC/100mL)	4333.33 ± 3669.69	5571.43 ± 8847.92	0.5975	28133.33 ± 24317.74	42349,17 ± 68845,04	0.3462

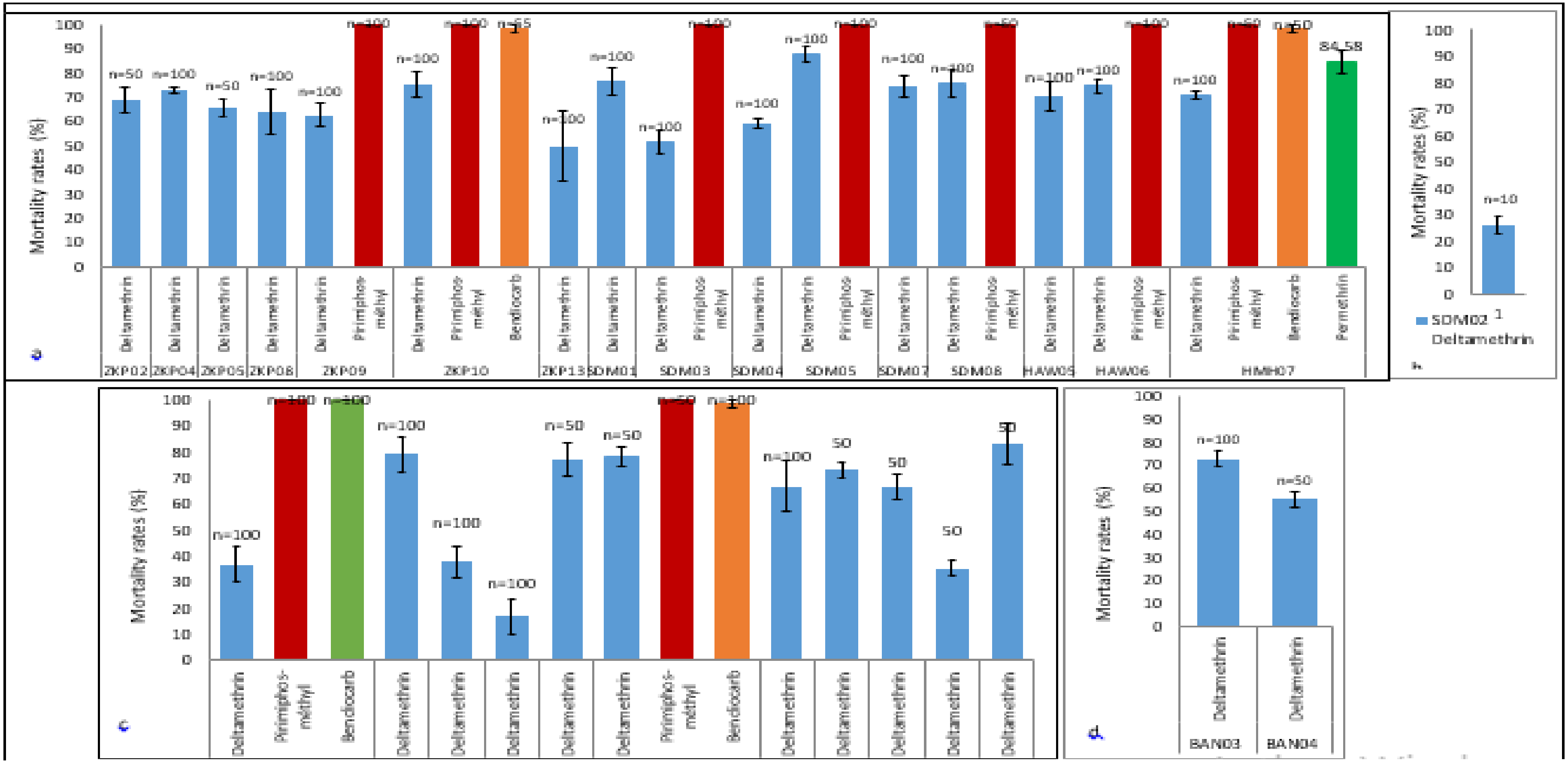


Figure 2: Insecticide resistance profiles of *Anopheles gambiae* s.l. populations in Bohicon (a: rainy saison ; b : dry season) and Parakou (c : rainy season ; d : dry season). ZKP : Zakpo ; SDM : Sodohomè ; HAW : Houawè ; HMH : Hominho ; BAN : Bannikani ; YKN : Yarakinin ; ABK : Albarika

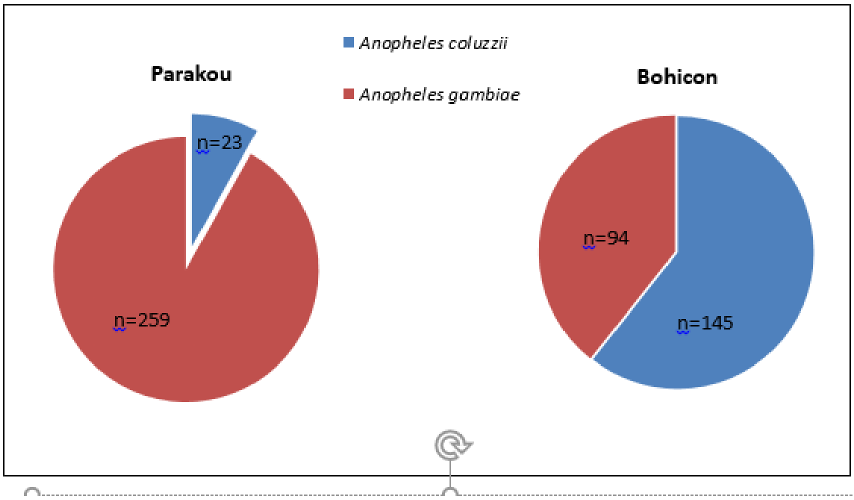


Figure 3: Distribution of sibling species of *A. gambiae* complex in both study sites.

Table 4: Mortality rate of *A. gambiae* s.l. from urban and non-urban areas of Bohicon and Parakou cities.

Insecticides		**Bohicon**		**Parakou**	
		Urban areas	**Non urban areas**	**Urban areas**	**Non urban areas**
Deltamethrin	Total tested (n)	600	900	400	550
	Mortality rate (%)	65.4	71	42.6	68.5
	P-value	0.0219		< 0.0001	
Pirimiphos-methyl	Total tested (n)	200	500	100	100
	Mortality rate (%)	100	100	100	100
	P-value	-		-	
Bendiocarb	Total tested (n)	55	60	50	100
	Mortality rate (%)	98.2	98.4	100	100
	P-value	0.9342		-	

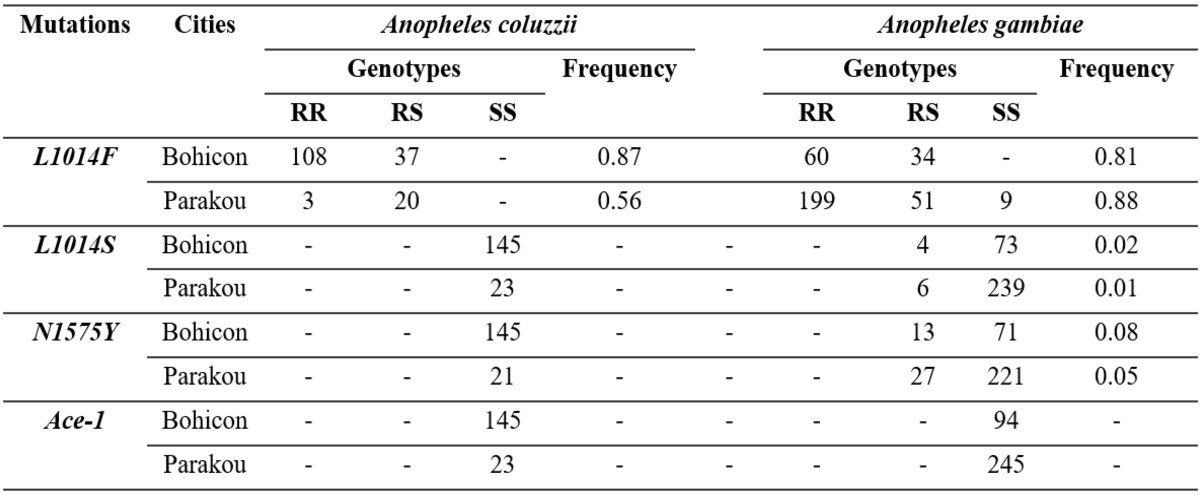
Table 5: Distribution of *A. gambiae* species according to degree of urbanization of the study sites.

	Anopheles coluzzii					***Anopheles gambiae***				
Sites	**Urban areas**		**Non-urban areas**		**Total**	**Urban areas**		**Non-urban areas**		**Total**
	N	%	N	%	**N (%)**	N	%	n	%	**N (%)**
Bohicon	145	100	-	-	**145 (100)**	94	100	-	-	**94 (100)**
Parakou	23	100	-	-	**23 (100)**	120	46.3	139	53.6	**259 (100)**

Table 6: Correlation between *Anopheles* species distribution and physico-chemical and microbial parameters of breeding sites.

Parameters	***A. gambiae***		***A. coluzzii***	
	r value	**P-value**	**r value**	**P-value**
Temperature (°C)	- 0.04	1	- 0.30	0.206
Ph	- 0.840	< 0.0001	- 0.63	0.024
Turbidity (NTU)	- 0.74	< 0.0001	- 0.23	0.268
Conductivity (µS/cm)	- 0.26	0.2014	- 0.53	0.037
Dissolved oxygen (mg/L)	0.51	0.002	0.60	0.0003
Salinity (g/L)	- 0.38	0.165	- 0.20	0.701
TDS (mg/L)	-0.10	0.490	- 0.59	0.0001
Total coliforms (UFC/100mL)	- 0.38	0.152	-0.10	0.1775
Fecal coliforms (UFC/100mL)	- 0.52	0.0019	0.02	1
E. coli **(UFC/100mL)**	0.01	1	-0.44	0.023

Table 7: Allelic frequencies of mutations within different species of the *A. gambiae* complex across the study cities.



Mutations	**Cities**	***Anopheles coluzzii***					***Anopheles gambiae***			
		Genotypes			**Frequency**		**Genotypes**			**Frequency**
		RR	**RS**	**SS**			**RR**	**RS**	**SS**	
L1014F	Bohicon	108	37	-	0.87		60	34	-	0.81
	Parakou	3	20	-	0.56		199	51	9	0.88
L1014S	Bohicon	-	-	145	-	-	-	4	73	0.02
	Parakou	-	-	23	-	-	-	6	239	0.01
N1575Y	Bohicon	-	-	145	-	-	-	13	71	0.08
	Parakou	-	-	21	-	-	-	27	221	0.05
Ace-1	Bohicon	-	-	145	-	-	-	-	94	-
	Parakou	-	-	23	-	-	-	-	245	-

Table 8: Correlation of physico-chemical parameters with the frequency of the *kdr L1014F* mutation.

Cities	Temperature	pH	Turbidity	Conductivity	Dissolved Oxygen	Salinity	TDS
Bohicon	0.906 (P = 0.039)	0.469 (P = 0.192)	- 0.066 (P = 0.402)	- 0.244 (P = 0.171)	0.409 (P = 0.20)	- 0.044 (P = 0.414)	- 0,281 (P = 0.182)
Parakou	- 0.482 (P = 0.196)	- 0.365 (P = 0.170)	0.191 (P = 0.211)	- 0.674 (P = 0.108)	0.4012 (P = 0.190)	- 0.924 (P = 0.022)	- 0.785 (P = 0.06)

DISCUSSION

The study investigated the breeding sites factors that influence the distribution of *A. gambiae* s.l. species, as well as their resistance to commonly used insecticides in the urban areas of Bohicon and Parakou. According to the analysis of results, high proportions of larval habitats were found in urban areas, notably in the city of Bohicon. Furthermore, a substantial proportion of these larval habitats had a human origin in both cities. This observation aligned with a growing body of research indicating the adaptation of Anopheles mosquitoes to the diverse breeding sites created by urbanization (Akorli et al., 2016). Indeed, urban areas often feature a proliferation of artificial containers such as discarded tires, abandoned containers, and other debris, which serve as ideal breeding sites for mosquitoes. Additionally, the increase of surfaces associated with urbanization, contribute to the formation of stagnant water pools, further increasing larval habitat availability. The accessibility and abundance of such habitats in urban settings, are significantly higher compared to non-urban areas due to factors like population density and infrastructure development.

Moreover, studies conducted in different urban settings provide further insights into the adaptability of *Anopheles* mosquitoes. Ossè et al. (2019) conducted a study demonstrating the adaptation of *A. coluzzii* in the polluted aquatic habitats of Cotonou. Further study conducted in Ghana, has also uncovered the presence of *A. gambiae* s.l. in environments that were once deemed unsuitable for mosquito breeding (Forson et al., 2023). This demonstrated the capacity of Anopheles mosquitoes to exploit a wide range of breeding sites in urban areas, contributing to the persistence of malaria transmission. As urbanization continues, it is crucial to consider the evolving dynamics of vector ecology and breeding habits, acknowledging the complex interplay between environmental changes and malaria transmission.

It is acknowledged that different species of mosquito prefer specific habitat water with diverse physico-chemical characteristics for their egg laying and larval survival (Seal et al., 2023).

Following the molecular analyses conducted in this study, it was observed that, despite coexisting in sympatry, there is a prevalence of *A. coluzzii* over *A. gambiae* in the city of Bohicon. Conversely, in Parakou, the analyses revealed a significant predominance of *A. gambiae*, constituting 91.9% of the analyzed mosquitoes. This distribution of the two species within the *Anopheles gambiae* complex is likely influenced by distinct ecological characteristics of larval habitats in these two cities, specifically their physicochemical properties (Ahadji-dabla et al., 2019). Indeed, in Bohicon a considerably lower temperature has been noted a compared to Parakou. The lower temperature in Bohicon may create conditions more favorable for the development and survival of *A. coluzzii*, while the warmer climate in Parakou might be conducive to the predominance of *A. gambiae*. Temperature is a crucial factor influencing the distribution of mosquito species, and this difference between the cities could contribute to the observed variations.

Furthermore, the concentrations of Total Dissolved Solids (TDS), conductivity, and salinity in Bohicon are lower than those observed in Parakou, with a p-value of less than 0.0001. This difference may be attributed to significant anthropogenic activities observed in Parakou, such as agriculture, industry, and urbanization, which can introduce chemicals and salts into water sources serving as larval habitats. Although the present analyses indicated that these parameters have no significant influence on the distribution of *Anopheles gambiae* specie. Anopheles species typically demonstrate preferences for breeding sites characterized by specific water chemistry traits. The disparity in these parameters between the two cities may still play a role in species-specific habitat selection (Mahamane Iro et al., 2020).

Conversely, in Bohicon, the result showed that the Turbidity is significantly higher than that observed in Parakou. Moreover, turbidity has a significant and negative impact on the presence of larvae of *A. gambiae* in breeding sites. These observations aligned with those of other authors who also emphasized the negative effect of turbidity on the presence of larval forms of Anopheles in aquatic habitats (Mwangangi et al., 2010). Several factors contribute to turbidity including insoluble particles of soil, organics, microorganisms, and other materials. The predominance of *A. coluzzii* in habitats with high turbidity in Bohicon could be explained by the remarkable adaptability of this species, as highlighted in several previous studies (Kweka et al., 2015).

The two sub-species of the *A. gambiae* complex identified in this study showed a significant positive correlation with the amount of dissolved oxygen in both cities. Other earlier studies consistently reported that a majority of Anopheles species prefer non-polluted water bodies characterized by high dissolved oxygen content for optimal larval survival (Dida et al., 2018 ; Afolabi et al., 2019).

Moreover, the results indicated that the larvae of *A. gambiae* were negatively associated with the presence of fecal coliform in larval habitats. As suggested by Paaijmans et al. (2010), microbial interactions can influence mosquito larval development. For instance, certain coliforms might outcompete or directly inhibit the growth of *A. gambiae* larvae, affecting their abundance and distribution. Microbial dynamics within breeding sites observed in this study, may therefore play a role in abundance and spatial distribution of Anopheles mosquito species.

The populations of *A. gambiae* tested showed high levels of resistance to deltamethrin and permethrin. The high level of resistance of these two malaria vectors to pyrethroids observed in Benin could be associated with environmental factors, such as pollution in these urban areas, which increase the level of xenobiotics in larval habitats (Aïkpon et al., 2020). Moreover, the observed resistance to pyrethroids may be attributed to several interconnected factors. Agricultural practices, including the extensive use of pesticides, have been implicated in insecticide resistance. Also, the intensive implementation of vector control interventions, predominantly relying on pyrethroid-based insecticides, could impose selective pressure, fostering the development of resistance (Hemingway et al., 2016).

The strong insecticide resistance observed in vectors was also associated with high frequencies of the *kdr L1014F* mutation. The frequencies were similar in *A. gambiae* and *A. coluzzii* at both study sites which suggested that the two species might have been under similar selection pressure. This result aligned with findings from Aïkpon et al. (2020), who similarly reported no significant difference in the *kdr L1014F* frequencies between *A. gambiae* and *A. coluzzii* in different regions of Benin. Moreover, results showed a correlation between the frequencies of the *kdr L1014F* mutation and key environmental factors. Specifically, these frequencies showed a significant correlation with temperature in Bohicon and agreed with the research conducted by Salinas et al. (2021), emphasizing the role of temperature in influencing the susceptibility to insecticides in Aedes species and possibly the prevalence of insecticide resistance mutations. In addition, a correlation was observed between the allelic frequencies of *kdr L1014F* and salinity in Parakou. Nevertheless, the involvement of salinity in the occurrence of the kdr mutation gene in *A. gambiae* remains insufficiently elucidated and warrants further investigation. This underscores the intricate interplay between genetic factors and environmental conditions in shaping insecticide resistance profiles among Anopheles populations.

The presence of the *kdr L1014S* resistance allele was identified in both surveyed sites, exclusively within a subset of mosquitoes belonging to *A. gambiae* species. The *kdr L1014S* mutation, had been previously characterized as the East African type of knockdown resistance. However, recent investigations have unveiled the occurrence of the *kdr L1014S* mutation in malaria vectors from West Africa, challenging the previously established geographic distribution (Hien et al., 2007). Furthermore, Djegbè et al. (2011) specifically identified this mutation in malaria vectors in Benin, contributing to the evolving understanding of the geographical distribution of the *kdr L1014S* mutation beyond its East African origins.

In this study, the *N1575Y* mutation was identified at a very low frequency in the two surveyed sites and was exclusively detected in the *A. gambiae* species. The existence of this mutation in Benin has been previously reported by Djègbè et al. (2014), and its presence has also been documented in other Sub-Saharan African countries such as Cameroon and Côte d'Ivoire (Fossog et al., 2013 ; Edi et al., 2017). The *N1575Y* mutation, situated within the voltage-gated sodium channel, exerts a synergistic effect on resistance to pyrethroids when co-occurring with the *kdr L1014F* mutation. This combination of mutations within the voltage-gated sodium channel not only enhances the resistance profile but also underscores the complexity of molecular mechanisms contributing to insecticide resistance in *Anopheles* populations (Wang et al., 2015).

This study provided encouraging findings by demonstrating the complete susceptibility of Anopheles mosquitoes to the pyrimiphos-methyl (organophosphate) and bendiocarb (carbamate) in the study sites. This susceptibility aligned with observations in other studies in the country (Kpanou et al., 2021; Yovogan et al., 2021) suggest that indoor residual spraying (IRS) using non-pyrethroid formulations may not be compromised in these sites.

Conclusion

This study challenges the notion that urbanization reduces malaria transmission risk by revealing significant Anopheles mosquito breeding in urban areas. The adaptability of mosquitoes to diverse urban breeding sites emphasizes the importance of understanding evolving vector ecology. The distribution of Anopheles species was influenced by ecological factors such as temperature, turbidity, and water chemistry. It highlighted prevalent insecticide resistance in *A. gambiae* s.l., especially to pyrethroids, linked to environmental factors and vector control practices. Genetic mutations associated with resistance showed a complex interplay with environmental conditions. However, the study revealed full susceptibility to non-pyrethroid insecticides, suggesting potential effectiveness in malaria control strategies. These findings emphasized the need for a nuanced and adaptable approaches considering both vector behavior and environmental dynamics in urban malaria control. It would be necessary to do insecticide resistance surveillance to Anopheles populations from Bohicon and Parakou.

COMPETING INTERESTS

The authors declare that they have no competing interest.

AUTHORS’ CONTRIBUTIONS

ID conceived and supervised the study. DHD, MKYGB, and ON carried out the field sampling and the recording of physicochemical parameters. ON and DHD conducted the microbiological analyses. DHD and GT carried out molecular analyses of sampled mosquitoes. DHD and MKYGB reared mosquitoes and performed WHO bioassays. DHD analysed all the data. SC, MA, LD, RD and FC provided advice during the design of the study. DHD wrote the manuscript, which has been critically reviewed for intellectual content by GT and ID. All authors have read and approved the final version of the manuscript.

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