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Malaria transmission and insecticide susceptibility of the anophelian fauna in the Njombé-Penja health district

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Abstract
The aim of the present study was to determine the dynamics of malaria transmission and the sensitivity of its vectors in order to better plan vector control programme. Mosquitoes captured on volunteers, from September 2019 to June 2020, at the rythm of 2 consecutive nights every three months, were identified according to morphological and molecular criteria. The infectivity of the vectors was determined by the ELISA CSP test. Insecticide susceptibility was assessed and resistance mechanisms were determined. The anopheles genus (64.07%; n=6020) was the most represented and *An. gambiae*, responsible for 82.61% of transmission in both sites, followed by *An. funestus* s.l. (17,39%). The average entomological inoculation rates were 2.03 ib/p/n at Njombé and 0.48 ib/p/n at Bonandam. Diagnostic PCR for resistance genes detected the presence of the kdr L1014F mutation in the natural population of *An. gambiae* s.l. High insecticide resistance requires the development of new mosquito control formulations.

Keywords: Anopheles, malaria transmission, insecticide resistance, njombe-penja, cameroon

1. Introduction
Malaria, due to its frequency and severity, remains the most important parasitic infection in public health. According to World Health Organization, 229 million cases of malaria and 409,000 deaths have been recorded worldwide [1]. Sub-Saharan Africa accounts for a significant and disproportionately large part of the global burden of the disease with over 94% of morbidity and mortality; the most vulnerable groups being children under 5 years of age and pregnant women. In Cameroon, this disease represents the main cause of medical consultation, hospitalization, morbidity and mortality [2, 3]. The public authorities devote an average of nearly 2 billion CFA francs per year to the fight against malaria, which therefore appears to be one of the main obstacles to the emergence that Cameroon has set itself as an objective to achieve by 2035 [4].
The State of Cameroon, supported by its development partners, has made the fight against this parasitosis its main battleground, essentially base on two approaches: one curative, through early diagnosis and the administration of anti-malarial drugs, and the other preventive, through anti-vectorial control and protection against vectors. The latter relies mainly on indoor residual spraying and the use of long-lasting insecticide-treated nets (LLINs) [1, 5].
The implementation of these measures has led to a reduction in malaria incidence more in some parts of the country than in others. The logic of standardising malaria control measures throughout the national territory without taking into account the specific ecological conditions encountered in Cameroon, would be at the origin of this situation. However, many studies have shown that the implementation of an effective vector control method should be subject to the availability, for each given eco-climatic facies, of reliable and updated entomological information that can account for the identity of vector mosquitoes, their role in malaria transmission and their susceptibility to the insecticides recommended by the WHO for vector control [6]. Such data are available in most localities in Cameroon and provide a basis for short- and medium-term evaluation of vector control operations. In the coastal region of Cameroon, for example, surveys conducted in the city of Douala and Yabassi showed that the vector system is predominantly composed of the species *Anopheles coluzzii* Coetzee & Wilkerson,

2013 and secondarily of other species such as *An. gambiae*, *An. melas*, *An. moucheti*, *An. nili* and *An. funestus*. The kdr resistance gene is partly responsible for the insensitivity of anopheles to insecticides recommended by the WHO [7, 8, 9, 10]. Apart from these localities which have aroused the curiosity of entomologists because of their ecological particularities, no other locality in this region of the country has been the subject of such studies.

The health district of Njombé-Penja is the main production area for food crops, mainly pepper (*Piper nigrum*). This is a condiment ingredient whose variety grown in Njombe-Penja is highly appreciated beyond the national and African borders. Almost 1/3 of the cultivable area in this locality is used for this crop. Recent studies have shown that the *Piper nigrum* plant has excellent insecticidal properties [11]. The essential oil of the fresh seeds of this plant is rich in β-Trans-caryophyllene, which is well known for its insecticidal potential (Ofono, pers. comm.). At a concentration of 200 ppm, this oil induces total mortality of stage 4 larvae of *An. gambiae* s.l. after 10 hours of exposure. It is therefore clear that mosquitoes in Njombe-Penja are permanently exposed to the insecticidal odors emitted daily by *Piper nigrum* plants. Thus, mosquitoes in the locality are not only subjected to the pressure of biological insecticides emitted by *Piper nigrum* plantations, but also to the pressure of chemical pesticides used by farmers to treat these plantations. This situation can have serious repercussions on the local anophelian fauna, the dynamics of malaria transmission and the resistance status of vectors to conventional insecticides.

The present study aims to determine the anophelian fauna, its role in malaria transmission and its susceptibility to insecticides in the Njombe-penja health district where 1/3 of the cultivable area is occupied by *Piper nigrum*.

2. Materials and Methods

2.1 Study site

The present study was conducted in the health district of Njombe-Penja (04° 34' N and 09° 39'E), a locality of about 36300 peoples [12] (Figure1). The climate is characterized by a long rainy season of nine months (March-November) and a short dry season of three months (December-February) [13]. The average annual rainfall is 2434.69 mm and the average annual temperature is about 26.5 °C [14, 15]. The hydrographic is made up of the Mungo and Dibombe rivers [15]. Mosquito collection took place in Njombé (urban site) and Bonandam (rural site).

Njombé (04° 34'N; 09° 39'E) is an urban site with progressively degraded vegetation due to human activities. Urbanization is poorly thought out. Most of the main and secondary roads have no gullies, causing flooding in the rainy season. The majority of dwellings are made of temporary materials (planks); the administrative and commercial infrastructures are made of permanent materials. The inhabitants practice extensive breeding (cattle and goats, poultry), domestic fishing and crafts. Agriculture is dominated by the cultivation of *Piper nigrum.* The locality has tens of hectares of plantations of this plant species. The tracks leading to these plantations have many puddles in the rainy season, which are potential temporary breeding grounds for anopheles, the cause of malaria cases in the locality. The vast majority of the population use LLINs to protect themselves from mosquito bites and go to a local health center in case of illness.

Bonandam (4° 35' N; 9° 40' E) is a village located about 4 km from the center of Njombé. Covering an area of 25 km^2, this village is mostly occupied by plantations within which a few traditional dwellings are drowned. The hydrographic network is very weak, consisting mainly of streams that wind through the village. Most of the water points in the locality as well as the puddles visible on the tracks during the rainy season constitute potential Anopheles breeding grounds. Suspected malaria cases are taken to neighboring localities, as Bonandam has no health center.

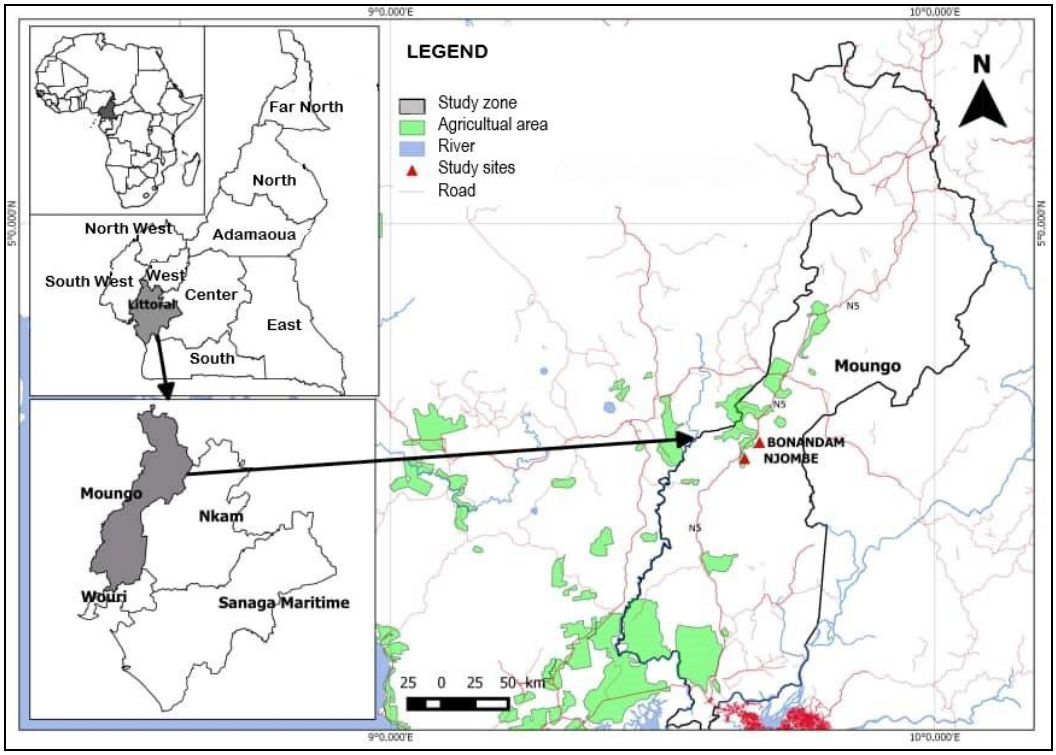

Fig 1: Location of study sites

2.2 Collection and treatment of Anopheles larvae

The Anopheles populations intended for the test were collected in the larval state in natural sites (puddles) using the dipping method [16]. The collection took place during the rainy season, from September to October 2019 at a rate of five consecutive days per month, simultaneously in Njombé and Bonandam. The collected Anopheles larvae were reared in water from the breeding sites and fed with Tetra Baby fish food [17]. The adults obtained were morphologically identified [18, 19] and adult females of *An. gambiae* s.l. aged 2-5 days old were tested to insecticide.

2.3 Collection and treatment of adult anopheles

Adult female mosquitoes were collected from September 2019 to June 2020. The sampling method used was nocturnal captures on volunteers. These captures took place from 6pm to 6am, at a rate of 2 consecutive nights per site, inside and outside 4 selected dwellings. The captured mosquitoes were identified on the basis of morphological criteria [19]. The ovaries of the anopheles were then dissected for physiological age determination based on the appearance of the ovarian tracheoles [20]. Each dissected anopheles was placed in a 1.5 millilitre Eppendorf tube containing silica gel and stored in a freezer at -20 °C for molecular analysis.

2.4 Laboratory analysis

Genomic DNA from mosquitoes was extracted following a protocol using 2% CTAB as grinding buffer [21]. The species of the *An. gambiae* complex were identified by SINE 200 PCR [22]. The determination of the infection rates of Anopheles was done after detection of the circumsporozoite protein (CSP) in the head and thorax using the CSP ELISA technique [23]. The search for the presence of the Kdr mutation in samples of insecticide-resistant Anopheles was done according to the protocol of Martinez-Torres *et al.* [24].

2.5 Statistical analysis

SPSS software (version 22.0, for Windows, SPSS Inc., Chicago, IL, USA) was used to perform statistical analyses. The Wilcoxon test was used for the comparison of aggressive densities between species, while the Mann-Whitney U test was used for the comparison of aggressive densities between indoor and outdoor dwellings. The WinDL50 software was used to determine the knockdown times (tkd_{50} and tkd_{95}) in Anopheles populations exposed to insecticides used in public health. The significance level for all statistical analyses was set at p=0.05.

2.6 Ethical consideration and informed consent

This study was approved by the ethics committee of the University of Douala. The purpose of the study was clearly explained to the populations of the study sites with the help of community agents and neighbourhood leaders. An informed consent form was voluntarily signed by each head of household approached. All participants in this study were previously vaccinated against yellow fever and subjected to malaria prophylaxis through the administration of 3 sulfadoxine-pyrimethamine 500 mg/25 mg tablets.

3. Results

3.1 Diversity of the culicidofauna in the Njombé-Penja health district

A total of 9396 adult mosquitoes were identified, 45.06% (n= 4234) from larval collections and 54.94% (n= 5162) from human landing captures. Individuals of the Anopheles genus (64.07%; n=6020) were the most represented followed by those of the Culex genus (29.92%; n=2811) (Table 1).

In Njombé, Culicidian fauna represented 63.43% of the total fauna of the Njombé-Penja health district. Of the 12 species identified, *An. gambiae* s.l (54.07%; n=3211) was the most abundant species, followed by *C. quinquefasciatus* (12.19%; n=724). In contrast, *C. culiciomaya*, *C. decens*, *C. duttoni* and *M. africana* were the least represented species (n<50) (Table 1).

In Bonandam, the Culicidian fauna represented 36.57% of the culicidofauna of the Njombé-Penja health district. *An. gambiae* s.l and *An. funestus* were the most abundant species with respectively 38.13% and 21.55% of the individuals identified. The least abundant species were *An. paludis*, *An. coustanii*, *C. culiciomaya*, *M. africana* (table 1).

Table 1: Species richness, abundance and aggressiveness rate of the culicidae fauna in the health District of Njombé-Penja

Culicidae fauna								
	Njombé				Bonandam			
	CHV (%)	LC (%)	Total (%)	ma (b/p/n)	CHV (%)	LC (%)	Total (%)	ma (b/p/n)
An. gambiae s.l	1334 (46.29)	1877 (61.40)	3211 (54.07)	29.35	817 (35.83)	501 (42.57)	1318 (38.13)	15.99
An. paludis	28 (0.97)	0(0.00)	28 (0.47)	0.75	24 (1.05)	4 (0.34)	28 (0.81)	0.55
An. funestus	449 (15.58)	224(7.33)	673 (11.33)	10.27	406 (17.81)	339 (28.80)	745 (21.55)	7.39
An coustanii	0 (0.00)	0(0.00)	0 (0.00)	0.00	17(0.75)	0 (0.00)	17 (0.49)	0.53
C. quinquefasciatus	514 (17.83)	210(6.87)	724 (12.19)	11.77	599 (26.27)	87 (7.39)	686 (19.84)	13.19
C. culiciomaya	32 (1.11)	11 (0.36)	43 (0.72)	0.69	29 (1.27)	0 (0.00)	29 (0.84)	0.71
C. poicilipes	126 (4.37)	82 (2.68)	208 (3.50)	3.27	48 (2.11)	17 (1.44)	65 (1.88)	1.22
C. decens	0 (0.00)	17 (0.56)	17 (0.29)	0.00	0 (0.00)	0 (0.00)	0 (0.00)	0.00
C. pipiens	287 (9.96)	395 (12.92)	682 (11.48)	5.60	254 (11.14)	72 (6.12)	326 (9.43)	5.41
C. duttoni	0 (0.00)	31(1.01)	31 (0.52)	0.00	0 (0.00)	0 (0.00)	0 (0.00)	0.00
Ae. aegypti	59 (2.05)	19 (0.62)	78 (1.31)	1.67	31(1.36)	51 (4.33)	82 (2.37)	0.90
Ae. albopictus	38 (1.32)	191(6.25)	229 (3.86)	1.01	50 (2.19)	106 (9.01)	156 (4.51)	1.24
M. africana	15 (0.52)	0 (0.00)	15 (0.25)	0.41	5 (0.22)	0 (0.00)	5 (0.14)	0.13
Total	2882 (100)	3057 (100)	5939 (100)	64.76	2280 (100)	1177 (100)	3457 (100)	47.26

CHV: Capture on human volunteers; LC: Larval collection; *An.*: *anopheles*; *Ae.*: *Aedes*; *M.*: *mansonia*; *C.*: *Culex*; ma: agression rate

3.2 Biting rate

The overall average aggression rate recorded in Njombé during this study was 64.76 b/H/n. Each inhabitant of the locality should receive 23737 bites per year. The most aggressive species were *An. gambiae* s.l and *C. quinquefasciatus* with 29.35 p/p/n and 11.77 b/p/n respectively. *M. africana* was the least aggressive species (0.41 b/p/n) (Table 1). *An. gambiae* s.l. was significantly more

aggressive outdoors than indoors (p=0.017). Peak aggression of *An. gambiae* s.l. and *An. funestus* occurred between 12 a.m. –2 a.m. (Figure 2).

In Bonandam, the overall mean aggression rate was 47.26 b/p/n. The most aggressive species were *An. gambiae* s.l (15.99 b/p/n) and *C. quinquefasciatus* (13.19 b/p/n). *M. africana* (0.13 b/p/n) was the least aggressive species to humans (Table 1). The aggressive densities were not significantly different between *An. gambiae* s.l and *C. quinquefasciatus*. However, a significant difference was observed between those of *An. gambiae* s.l and the other species collected ($p<0.05$). The peak of aggressiveness of *An. gambiae* s.l. was in the time range 10 p.m. -12 a.m. time while that of *An. funestus* was in the range 2:00-4:00 h (Figure 3).

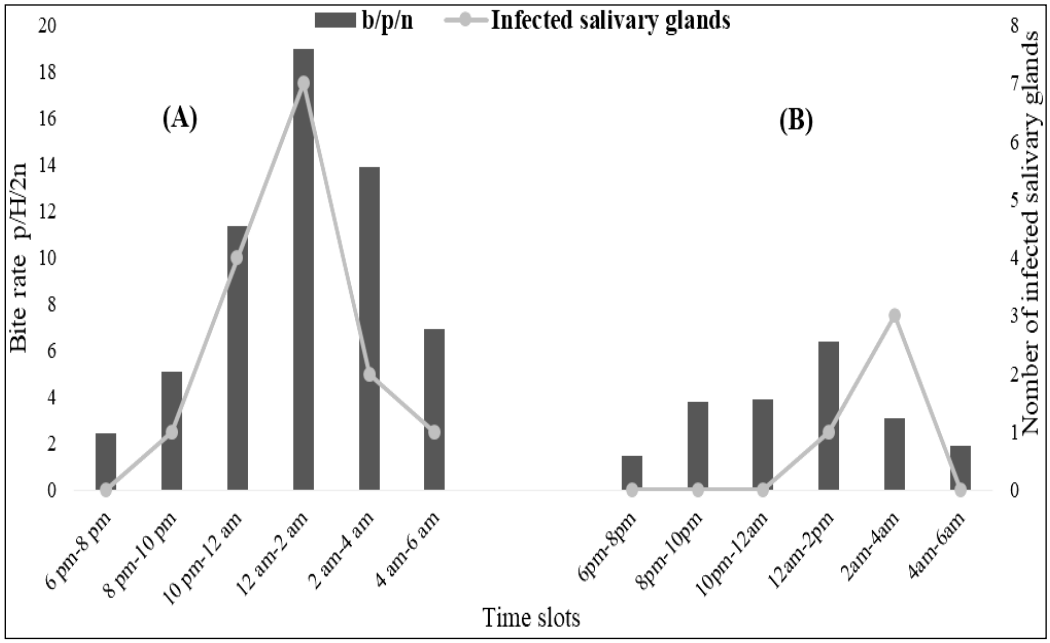

Fig 2: Cycle of aggressiveness and infectivity of *An. gambiae* s.l and *An. funestus* in Njombé

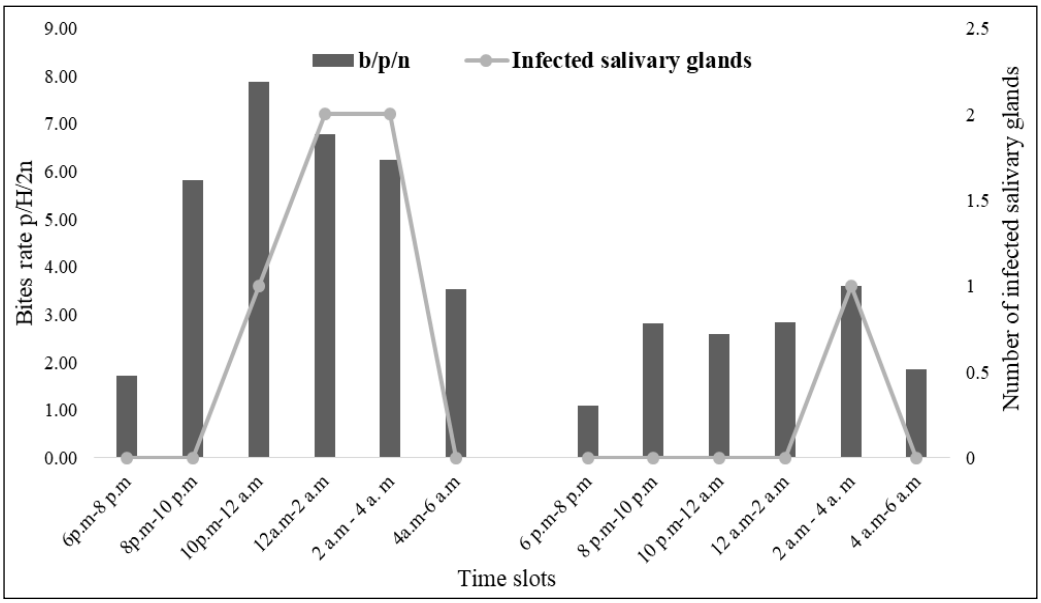

Fig 3: Cycle of aggressiveness and infectivity of *An. gambiae* s.l and *An. funestus* in Bonandam

3.3 Parturition rates, infection rates and malaria transmission

In Njombé, the average parity rates were 72.34% for *An. gambiae* s.l. (353/488 dissected females), 72.58% for *An. funestus* s.l. (45/62) and 46.43% for *An. paludis* (13/28). These rates were higher in the dry season than in the rainy season (Tab. 2). In Bonandam, the average parity rates were 75.96% for *An. gambiae* s.l. (256/337 females dissected), 64.29% for *An. funestus* s.l. (90/140), 41.67% for *An. paludis* (10/24) and 52.94% for *An. coustanii* (9/17). These rates were also higher in the dry season than in the rainy season (Table 2).

A total of 25 anopheles specimens out of 609 tested by ELISA-CSP were found to be infected with *Plasmodium falciparum* in the two study sites, representing an overall annual infectivity rate of 4.11% and an average annual entomological inoculation rate (EIR) of 889.06 pi/H/yr. This EIR was 2.03 ib/p/n in Njombé and 0.48 ib/p/n in Bonandam. *An. gambiae* s.l. was responsible for 82.61% of the transmission in both sites, followed by *An. funestus*. (17.39%) (Table 2).

Table 2: Seasonal variations of parturity rate, entomological inoculation rates and sporozoite indices of malaria vector in the health district of Njombé-penja

		Njombé			Bonandam			
		An. gambiae	***An. funestus***	***An. paludis***	***An. gambiae***	***An. funestus***	***An. paludis***	***A. coustanii***
Rainy season	Dissected	307	82	8	201	100	13	0
	Parity rate (%)	64.5	69.51	37.50	73.13	55.00	23.08	0.00
	SI	0.04	0.00	0.00	0.01	0.00	0.00	0.00
	EIR (ib/p/n)	0.99	0.00	0.00	0.19	0.00	0.00	0.00
Dry season	Dissected	181	22	20	136	40	11	17
	Pares (%)	85.64	81.82	50.00	80.15	87.50	63.64	52.94
	SI	0.06	0.18	0.00	0.06	0.03	0.00	0.00
	EIR (ib/p/n)	2.03	2.34	0.00	0.77	0.13	0.00	0.00

SI: Sporozoic index; EIR: Entomological inoculation rate.

3.4 Sensitivity and resistance genes of *An. gambiae* s.l. to insecticides

A total of 760 fasting adult female *Anopheles gambiae* s.l. mosquitoes aged two to five days were subjected to diagnostic doses of three insecticides used in public health (DDT 4%, Deltamethrin 0.05% and Permethrin 0.75%). This included 240 mosquitoes from the sensitive laboratory strain, 280 mosquitoes from the locality of Njombé and 240 mosquitoes from Bonandam. The tkd_{50} and tkd_{95} of the *An. gambiae* s.l populations in Njombé and Bonandam were well above 60 minutes. In contrast, the tkd_{50} and tkd_{95} of the reference susceptible populations (Kisumu strain) were between 9 and 30 minutes (Table 3). In Njombé, mortality rates were 32.5%, 38% and 15% respectively in the presence of deltamethine, permethrin and DDT. These mortality rates were similar to those obtained for the same insecticides in Bonandam (Table 4).

Diagnostic PCR for resistance genes detected the presence of the kdr L1014F mutation in the natural population of *An. gambiae* s.l. In the Njombé locality, only the resistant allele (R) that offers cross-resistance to DDT/Pyrethroid has been identified (Table 5). Therefore, all individuals diagnosed were genotypically resistant (RR) with a genotypic frequency of 100%. In contrast, individuals carrying both susceptible and resistant alleles were identified in Bonandam, however, the R allele was the most prevalent with a frequency of 0.975 in the rainy season and 1 in the dry season (Table 5).

Table 3: Knock-down times of the Kisumu *Anopheles gambiae* s.s. and the natural strain of *Anopheles gambiae* s.l to insecticides

Strain	Insecticide	N	KDT_{50} (mn) [CI_{95}]	KDT_{95} (mn) [CI_{95}]
Njombé	Delta 0.05%	80	> 60	> 60
	Perm 0.75%	100	> 60	> 60
	DDT 4%	100	> 60	> 60
Bonandam	Delta 0.05%	80	> 60	> 60
	Perm 0.75%	80	> 60	> 60
	DDT 4%	80	> 60	> 60
Kisumu	Delta 0.05%	80	11.2 [10.4-12.1]	17,7 [16.4-19.0]
	Perm 0.75%	80	9.0 [7.4-10.5]	19,6 [17.2-22.0]
	DDT 4%	80	22.9 [21.5-24.3]	29,1 [24.9-33.3]

N: sample size tested; tkd_{50}: knock-down time; CI_{95}: confidence interval at 95%; mn: minute; Delta: deltamethrin; Perm: permethrin; KDT_{50} et KDT_{95}: time required to knock-out 50% and 95% of mosquitoes.

Table 4: Mortality rates of Kisumu *Anopheles gambiae* s.s. and wild *Anopheles gambiae* s.l from Njombé and Bonandam 24 h post- exposure to 4% DDT, 0.75% permethrin or 0.05% deltamethrin

Strain	Insecticide	N	Mortality (%)	Control	Status
Njombé	Delta 0.05%	80	32.5%	0	R
	Perm 0.75%	100	38%	0	R
	DDT 4%	100	15%	0	R
Bonandam	Delta 0.05%	80	36.25	0	R
	Perm 0.75%	80	32.5%	0	R
	DDT 4%	80	21.25%	0	R
Kisumu	Delta 0.05%	80	100%	0	S
	Perm 0.75%	80	100%	0	S
	DDT 4%	80	95%	0	S

N: Number tested; Delta: deltamethrin; Perm: permethrin; R: Resistant; S: Sensitive

Table 5: Allelic and genotypic frequencies of the kdr L1014F mutation

Frequencies							
			genotypic			allelic	
Sites	**Season**	**N**	**RR (f)**	**RS (f)**	**SS (f)**	**f (R)**	**f (S)**
Njombé	Rainy	60	60 (100%)	0 (00%)	0 (00%)	1	0
	Dry	35	35 (100%)	0 (00%)	0 (00%)	1	0

Bonandam	Rainy	40	38 (92%)	2 (8%)	0 (00%)	0.975	0.025
	Dry	35	35 (100%)	0 (00%)	0 (00%)	1	0
Total	Rainy	100	98 (98%)	2 (2%)	0 (00%)	0.99	0.01
	Dry	70	70 (100%)	0 (00%)	0 (00%)	1	0

RR= homozygous resistant; RS= heterozygous; RR= homozygous susceptible; f(R) = frequency of the resistant allele = [nRS + 2x (nRR)]/2N; f(S) frequency of the sensitive allele = [nRS + 2x (nSS)]/2N.

4. Discussion

Entomological surveys conducted in the Njombe-penja health district revealed an abundant and diverse culicidofauna, whose spatio-temporal distribution and trophic behaviour are closely related to the eco-climatic characteristics of each capture site.

In Njombé, *An. gambiae* s.l was the most abundant species, followed by *Culex quinquefasciatus* and *Culex pipiens*. These results contrast with those obtained in several studies conducted in southern Cameroon which show that, in relation to the ecological characteristics favourable to their proliferation, species of the genus Culex are the most abundant in urban areas [25, 26, 27]. This predominance of *An. gambiae* s.l. would be due to the presence in the study site of water collections conducive to the development of this Anopheles species. Indeed, the tracks leading to the *Piper nigrum* fields present numerous collections of water, shallow, more or less sunny, clear and provided or not with an upright vegetation. Numerous studies in central Africa show that *An. gambiae* s.l. larvae are dependent on such sites [28]. Studies conducted in parallel to these in the same site indicate that these larvae were also found in sites with fairly high physicochemical parameters (electrical conductivity, TDS and pH) (Offono, pers. comm.), proof of the adaptation of this species complex to the environmental conditions imposed by the increasing urbanisation of cities [29]. According to several studies, *An. coluzzii*, is the species of the gambiae complex which seems to adapt best to the polluted environments of urban sites [30, 31, 32, 33]. This information is all the more true as identification using molecular techniques carried out on a sample of *An. gambiae* s.l from Njombé, revealed the unique presence of *An. coluzzii* Coetzee & Wilkerson (Ofono, pers. com).

Culicidian diversity in Bonandam is with a few exceptions, similar to that observed in Njombé. A total of 11 species of mosquitoes were identified, of which *An. gambiae* s.l, *An. funestus* and *Culex quinquefasciatus* were the three most abundant species respectively. The diversity observed is closely related to the multitude of breeding sites encountered in this study site. The presence of a greater number of species of the genus Anopheles testifies to the much more natural character of the Bonandam locality. This observation tends to be justified by the presence in the locality of species that are adapted to forest environments such as *An. coustani* and *An. paludis*. The latter are fond of collections of natural, clear, slightly salty water, rich in floating, upright or hydrophytic vegetation. Stream margins, ditches and flood depressions are their preferred sites [34].

Aggressive densities of Anopheles (*An. gambiae* s.l and *An. funestus*) fluctuated with the seasons and were found to be higher in the rainy season than in the dry season in all study sites. Indeed the rains offers a multiplicity of water collections, potential breeding sites for female mosquitoes, compared to the dry season when most sites are more or less dry. Furthermore, in our study sites, rainfall during the rainy season was sufficiently spaced out in time, allowing Anopheles to complete their development cycle in time, generally before the breeding sites were washed away. This result is in line with those presented by several authors who carried out similar work in sub-Saharan Africa [35, 36, 37].

Except for *An. funestus* which had a strong tendency to endophagy, the majority of the other species caught in the sites considered were exophagous, preferentially biting humans outdoor than indoor. This strong tendency towards exophagy, which is increasingly noticeable in Central Africa, may be explained by the high rate of ownership and use of insecticide-treated bed nets in households. In addition to acting as a physical barrier, limiting host/vector contact, LLINs also provide chemical protection through the excito-repellent action of alphacypermethrin, an insecticide of the pyrethroid family, which constrains mosquitoes to adopt exophagous behaviour [28].

In addition, the use of insect repellents and spray in some homes in the study sites could also justify for the exophagous behaviour of these mosquitoes. Conversely, it could be suggested that only mosquito species that have developed some genetic or metabolic resistance to this insecticide would adopt endophagous behaviour [38].

The aggression cycles of *An. gambiae* s.l and *An. funestus* show maximum activity between 10 p.m. and 4 a.m. This part of the night corresponds to the hours of deep sleep when a human, asleep, is easy prey liable to receive bites and be infected. This situation brings the issue of the quality and effective use of ITNs. Similar observations have been made in several localities in South Cameroon [7, 10, 39]. However, our results contrast with those of Mbida *et al*. [9] (2016) in Manoka, a small island in the city of Douala that is mainly used for fish farming. In this locality, the peak of aggressiveness of *An. coluzzii*, was located in the time slot 4 a.m - 06 a.m in the morning, corresponding to the time when fishermen are back from their fishing activity. This situation clearly reflects the ability of these malaria vectors to adapt to human habits in order to maximize their chances of having a blood meal. It would therefore be advisable, on the one hand, to increase awareness campaigns on the proper use of mosquito nets during sleeping hours, and on the other hand, to combine these with additional protective measures during or outside sleeping hours to achieve maximum protection for the population.

These hours of high culicidal aggressiveness also corresponded to the hours of high infectivity of *An. gambiae* s.l and *An. funestus*, the main malaria vectors in the Njombe-penja health district. Indeed, most of the infected salivary glands were recorded around the hours of 10 p.m-4 a.m in the morning. Infectivity rates, as well as parity rates of these two vectors were higher in the dry season than in the rainy season. This would be justified by the fact that the density of vectors in the dry season tends to decrease due to the scarcity of oviposition sites and the harsher climatic conditions. Furthermore, this suggests that female anopheles live longer in the dry season, which allows the parasite to complete its life cycle. This extension of the lifespan of female Anopheles results to an increase in the individual vectorial capacity of the survivors [37, 40]; hence the high entomological inoculation rates during this period of the year.

The results of sensitivity tests to discriminatory doses of

conventional insecticides show a high resistance of local *An. gambiae* s.l. populations to different insecticide doses (DDT 4%; permethrin 0.25% and deltamethrin 0.05%). This resistance is all the stronger as the knock-down times (Tkd_{50} and Tkd_{95}) of the local populations of *An. gambiae* s.l. are higher than 60 minutes for all the insecticides tested contrary to the sensitive laboratory strain in which these values are less than 30 minutes. The emergence of resistance to pyrethroid/DDT in local populations of *An. gambiae* s.l. would not only be the consequence of a high exposure of these populations to these insecticides during both disinsection and malaria control campaigns undertaken around the 1960s-1970s [31, 32, 41], but also to the establishment of crop protection programs against insect pests as observed in the Njombé and Bonandam plantations. The cross-resistance to pyrethroids/DDT observed in *An. gambiae* s.l in Njombé and Bonandam is associated not only with the modification of the voltage-dependent sodium channel but also probably with the increase in metabolic detoxification activity [42]. Indeed, the Kdr mutation, responsible for resistance to DDT and pyrethroids, is the main resistance mechanism identified in *An. gambiae* s.l. populations. The allelic frequency of this mutation is very high in both study sites, which constitutes a real problem insofar as the Kdr gene associated with metabolic resistance mechanisms could have a significant impact on the effectiveness of vector control tools associated with chemicals of the pyrethroids and organochlorines family [43]. These results are in agreement with those obtained by several authors [31, 41, 44] who noted an increasing evolution of this resistance over the years with a tendency to fix the resistant allele (L1014F) in the populations of *An. gambiae* s.l.

5. Conclusion

The present research shows that malaria vectors are present in Njombé and Bonandam. They are resistant to insecticides and maintain malaria transmission throughout the study period notwithstanding the protective measures in place. Awareness campaigns on the proper use of LLINs and the association of this tool with other complementary preventive measures are urgent measures to be recommended if we want to significantly reduce the prevalence of malaria in these agricultural sites.

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