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Characterization of Species of the *Anopheles gambiae* Complex in Kintele and Djoumouna (Brazzaville, Congo)

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Abstract

The effectiveness of implementing vector control strategies for malaria is assessed by monitoring vector dynamics and their role in malaria transmission. This study was carried out between September 2016 and August 2017 in Kintele and Djoumouna, and in August 2019 in Djoumouna alone. The objective of this study was to identify the *Anopheles* species involved in malaria transmission, their dynamics over time, and to evaluate the entomological indices based on the types of bedrooms investigated. *Anopheles* mosquitoes were collected manually from the residual fauna in the morning. Entomological indices were determined following the dissection of the *Anopheles* mosquitoes. Collections were performed in 541 bedrooms. The collected *Anopheles* species included *Anopheles gambiae s.l.* (1130), *Culex quinquefasciatus* (681), *Mansonia uniformis* (5), *Aedes albopictus* (1) and *Anopheles funestus* (1). Out of the 343 female *An. gambiae s.l.* mosquitoes whose DNA was amplified by PCR, the identified species from the *An. gambiae s.l.* complex consisted of *An. gambiae* (248), *An. coluzzii* (93), *An. arabiensis* (1) and one hybrid (1). These species were shown to be involved in malaria transmission, with higher intensity observed in sleeping rooms without Long-Lasting Insecticide Impregnated Mosquito Net (LLINs), particularly in Djoumouna. In these bedrooms, an individual received an average of 1 infectious bite every 27 days. Monitoring the status of LLINs and vectors proved to be necessary for maintaining knowledge about the effectiveness of LLINs.

Subject Areas

Immunology, Infectious Diseases

Keywords

Malaria, *An. gambiae*, *An. coluzzii*, *An. arabiensis*, Hybrid, Transmission, LLIN, Vector, EIR, Congo

1. Introduction

Malaria remains the most widespread parasitic disease globally, despite extensive efforts aimed at its elimination. In the year 2023 alone, worldwide, there were 263 million reported cases of malaria worldwide, resulting in 597,000 deaths [1].

In the Republic of Congo, malaria is a significant public health challenge. For the year 2021, a total of 1 327 964 malaria cases were recorded [2]. The primary vectors responsible for transmitting malaria in this region belong to the *An. gambiae* complex that comprises nine cryptic species [3] [4]. Among these, three species are considered major vectors: *An. gambiae s.s.*, *An. coluzzii* and *An. arabiensis* [5]. These species exhibit notable levels of attraction to humans (anthropophily) and preference for indoor environments (endophily). Additionally, their remarkable adaptability to human settlements and surroundings establishes them as some of the most proficient carriers of malaria worldwide. Studies conducted within the Republic of Congo have indicated that species belonging to the *An. gambiae s.l.* complex contribute to over 90% of malaria transmission [6].

Across various regions in Africa, these species live in sympatry. Hence, accurate identification of these species is imperative for studying their biological and ecological characteristics, such as relative abundance, population dynamics, and their roles in malaria transmission. This information is vital to gather the necessary data for implementing targeted control strategies [7] [8].

The present study was conducted in this context to identify the different species involved in malaria transmission in the urban locality of Kintele and the rural locality of Djoumouna and to evaluate it based on the different types of investigated bedrooms.

2. Material and Methods

2.1. Study Sites

The selection of study sites was based on previous entomological surveys and the degree of urbanization.

The commune of Kintele (4˚9'0"S, 15˚20'32"E) is situated 25 km north of Brazzaville, with an average altitude of 201 m. The estimated population is 11,105 inhabitants. Economic activities are primarily supported by small and medium-sized enterprises and state-owned markets. Housing structures consist of bricks buildings with sheet metal roofs. The relief is made up of valleys, plains, hills and

plateaus. The climate is tropical and humid, characterized by distinct wet (October to May) and dry (June to September) seasons. Annual average rainfall and temperature are 1370 mm and 25.5˚C, respectively. Kintele is intersected by two rivers, the Djiri and the Blue Chatelet. The predominant vegetation is savannah trees, sometimes accompanied by shrubs [9]. A study conducted in 1987 on malaria transmission indicated that the *Anopheles gambiae s.l.* complex contained the primary malaria vector species in Kintele, namely *An. gambiae s.l.* and *An. funestus* [10].

The village of Djoumouna (4˚22'34''S, 15˚9'36''E) is located 25 km southeast of Brazzaville in a rural area at an average altitude of 217 m [11]. The estimated population is 635 inhabitants. Main activities in this region encompass agriculture, fish farming and subsistence livestock. Dwellings are clustered and constructed from cement or clay bricks, with sheet metal and cob roofs. Djoumouna is located within a region of degraded secondary forest. The climate is humid tropical, marked by a rainy season (October to May) and a dry season (June to September). Data provided by the National Civil Aviation Agency between September 2016 and August 2017 [12] indicates average monthly rainfall and temperature of 134 mm and 26.1˚C, respectively, with an average maximum relative humidity of 90%. Djoumouna is bordered by four rivers: Djoumouna, Kinkoue, Lomba, and Loumbangala, which contribute water to a network of fish ponds. These ponds are periodically emptied for fish recovery or when irrigation canals are obstructed, creating stagnant water pools conducive to malaria vector proliferation. Furthermore, natural sites sustain *Anopheles* population year-round. The vegetation is composed of diverse species from to different plant families [13]. Extensive studies were conducted in the village between the 1970s to the 1990s [14] [15], which showed that species within to the *An. gambiae s.l.* complex were the principal malaria vector species of malaria. Regarding insecticide resistance, *An. gambiae s.l.* species were sensitive to deltamethrin [16].

2.2. Sampling and Processing of Culicidae

Adult culicids were collected in Kintele and Djoumouna (See **Figure 1** and **Figure 2**) between September 2016 and August 2017, and in August 2019 in Djoumouna alone. Collections were conducted monthly, over 3 consecutive days in each locality. The bedrooms were categorized based on the absence or presence of LLINs and their condition (good or torn). These LLINs were sourced from the distribution carried out by the NMCP between 2011 and 2012, as well as from LLINs sold in markets and distributed through campaigns in the DRC.

The collection method used was the manual collection of residual fauna in bedrooms [17]. The collected *Anopheles* mosquitoes were used entomological assessment.

Morphological identification of the specimens was conducted at the genus and species levels using the dichotomous keys developed by Edwards [18] and Gillies and De Meillon [19]. After collection, specimens were placed in individual tubes containing silica gel and cotton, subsequently stored at −20˚C for further analysis.

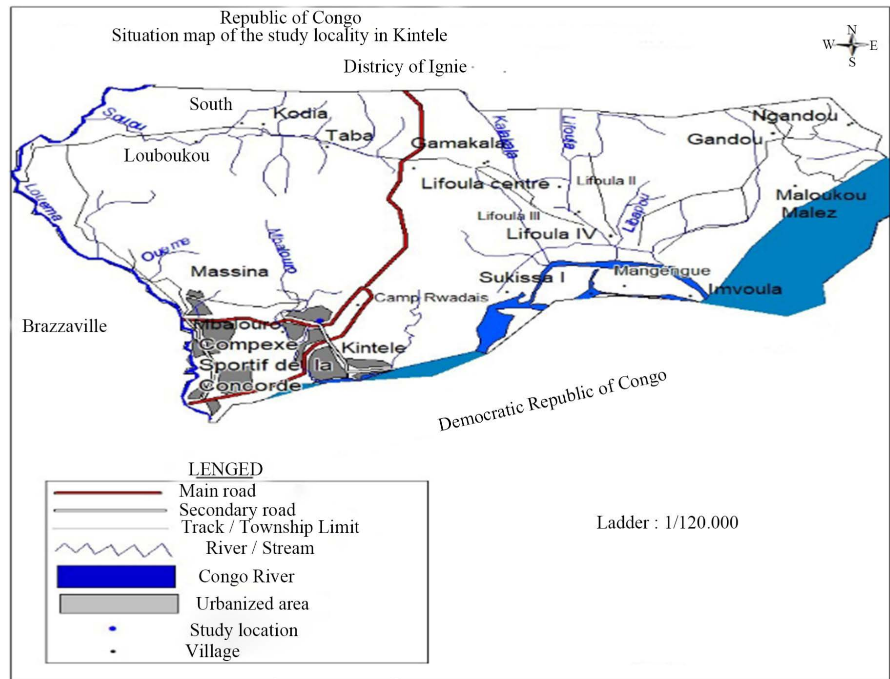

Figure 1. Map of Kintele with the location of adult collection sites (source: CERGEC 2020).

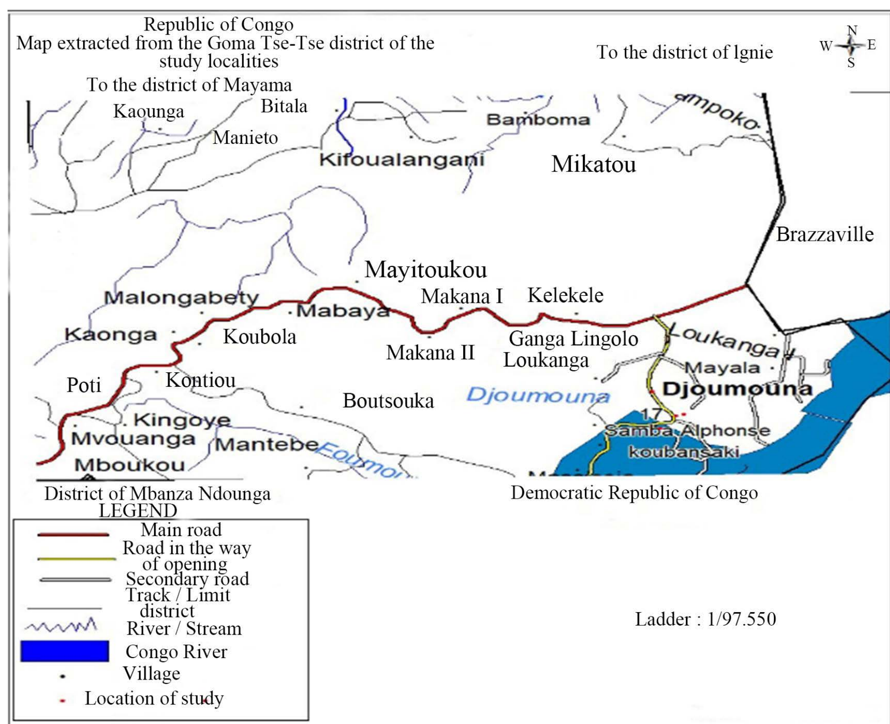

Figure 2. Map of Djoumouna with location of adult collection sites (source: CERGEC 2020).

2.3. Processing in the Laboratory

2.3.1. DNA Extraction

DNA was extracted from mosquitoes preserved with silica gel using the protocol

described by Morlais *et al.* [20]. The procedure involved grinding the legs and wings of the mosquitoes in 200 µl of 2% CTAB (Cetyl Trimethylammonium Bromide) in an Eppendorf tube. Subsequently, the tubes were incubated at a minimum of 65˚C for 5 minutes using a dry bath, followed by a 3-minutes cooling period on ice. To each tube, 200 µl of chloroform was added. After manually shaking the tubes, they were centrifuged at 12,000 rpm for 5 minutes. The supernatant from each tube was carefully transferred to a new tube, to which 200 µl of isopropanol was then added. The new tubes were subsequently centrifuged at 12,000 rpm for 15 minutes, and after that, the supernatant was discarded, leaving the DNA pellet. This pellet was washed with 200 µl of cold 70˚C ethanol, followed by centrifugation at 12,000 rpm for 5 minutes. The tubes were then emptied of ethanol and placed in a speed-vac to allow the DNA pellet to dry for 5 minutes. The resulting DNA was dissolved in 20 µl of bi-distilled water and stored at −20˚C until PCR testing.

2.3.2. Identification of the *An. gambiae* Complex Species by PCR

The molecular identification of species belonging to the *An. gambiae s.l.* complex was carried out following the method developed by Wilkins *et al.* [21]. PCR was performed using Thermus aquaticus polymerase from Sigma, USA. The reaction volume was 12.5 µl, consisting of the following components: 6.4 µl of water; 1.25 µl of 10× buffer; 1.25 µl of 2 mM dNTP; 0.5 µl of each primer (UN, AR, ML, M1, and S1); 0.1 µl of Taq polymerase, and 1 µl of DNA template.

The PCR reaction mixture was initially incubated at 95˚C for 5 minutes, followed by 30 amplification cycles: denaturation at 95˚C for 30 seconds, hybridization at 58˚C for 30 seconds, and elongation at 72˚C for 30 seconds. A final elongation step was performed at 72˚C for 5 minutes.

The size of the amplified DNA fragments was assessed through electrophoresis on a 2% agarose gel. The agarose gel was prepared by dissolving 2 g of agarose in 100 ml of 0.5× TBE buffer, supplemented with 5 µl of ethidium bromide (BET). The gel was then subjected to electrophoresis at 170 volts for 20 minutes. Visualization was done under UV light. The sizes of the resulting bands were determined using a 1 kb DNA ladder as reference; 221 bp for *An. gambiae*, 333 bp for *An. coluzzii* and 387 bp for *An. arabiensis*.

2.4. Statistical Analysis

In this study, data were collected in a Microsoft Office Excel 365 file, subsequent analyses were conducted using R software (version 8.4.1). Figures were generated with GraphPad-Prism software (version 3.6.3). For categorical variables involving *Anopheles* species, bedroom types and localities, proportions or percentages were calculated. To provide a measure of certainty, the binomial method was applied to establish the 95% confidence intervals for these parameters. Comparisons of proportions were carried out using the statistical tests such as Pearson chi-square (KHI-2) and Fisher's exact. When dealing with anopheline densities equal to or greater than 5, nonparametric including the Kruskal-Wallis and Mann-Whitney

tests were used. For means comparisons, the one-way ANOVA method was used, along with the and the Kruskal-Wallis test. To ascertain whether one locality exhibited a significantly higher percentage of *An. coluzzii* compared to another, the Odds Ratio was used with a 95% confidence interval.

3. Results

During this study, 269 houses were visited in Kintele, and 101 in Djoumouna. A total of 75 collection sessions were conducted to collect residual fauna from 541 bedrooms. These bedrooms were inhabited by 2382 individuals on the night before the collections.

3.1. *Anopheles* Densities by Bedroom Type in Both Locations

A total of 1818 female mosquitoes were collected, comprising four genera: 1131 *Anopheles*, 681 *Culex* (with *Culex quinquefasciatus*), 5 Mansonia (*Mansonia uniformis*) and 1 Aedes with (*Aedes albopictus*). Among the *Anopheles* mosquitoes, 1130 (99.9%) were females belonging to the *Anopheles gambiae s.l.* complex, and 1 female (0.1%) belonged to the *Anopheles funestus* group.

Table 1. Monthly distribution of *Anopheles gambiae s.l.* complex females in Kintele and Djoumouna (September 2016 to August 2017, and August 2019 in Djoumouna alone).

Months	Bedrooms without LLIN		Bedrooms with LLIN in good condition		Bedrooms with LLIN torn		Total of *An. gambiae s.l.*
	An. gambiae s.l. number	Number bedroom	*An. gambiae s.l.* number	Number bedroom	*An. gambiae s.l.* number	Number bedroom	
Sept.-16	**2**	6	**0**	19	**70**	21	**72**
Oct.-16	**4**	4	**2**	18	**101**	13	**107**
Nov.-16	**0**	0	**17**	19	**82**	15	**99**
Dec.-16	**0**	0	**9**	16	**81**	21	**90**
Jan.-17	**6**	9	**12**	26	**70**	22	**88**
Feb.-17	**26**	7	**13**	25	**81**	23	**120**
Mar.-17	**16**	14	**7**	19	**61**	16	**84**
Apr.-17	**26**	4	**10**	34	**77**	11	**113**
May-17	**38**	7	**12**	14	**21**	11	**71**
Jun.-17	**5**	6	**5**	13	**36**	19	**46**
Jul.-17	**12**	9	**1**	13	**29**	27	**42**
Aug.-17	**9**	7	**4**	13	**95**	27	**108**
Aug.-19	**35**	3	**1**	3	**54**	7	**90**
Total	**179**	76	**93**	232	**858**	233	**1130**

Furthermore, within the *An. gambiae* complex 136 (12.04 %) females were collected in Kintele and, and a larger number of 994 (87.96 %) in Djoumouna.

The statistical analysis indicated a significant difference between the collection localities and the diversity of collected species ($\chi^2 = 1301.2$; p-value < 0.001).

The monthly distribution of *An. gambiae s.l.* females, categorized by bedroom type, is detailed in Table 1.

Table 1 shows the number of females collected based on bedroom types in both locations. The highest average density was collected in bedrooms with torn LLINs (3.68 females), while the lowest density was found in bedrooms with LLINs in good condition (0.40 females). In November and December 2016, no dwellings with bedrooms lacking LLINs were visited.

Variations in population densities of the *An. gambiae* complex are shown in Figure 3 and Figure 4.

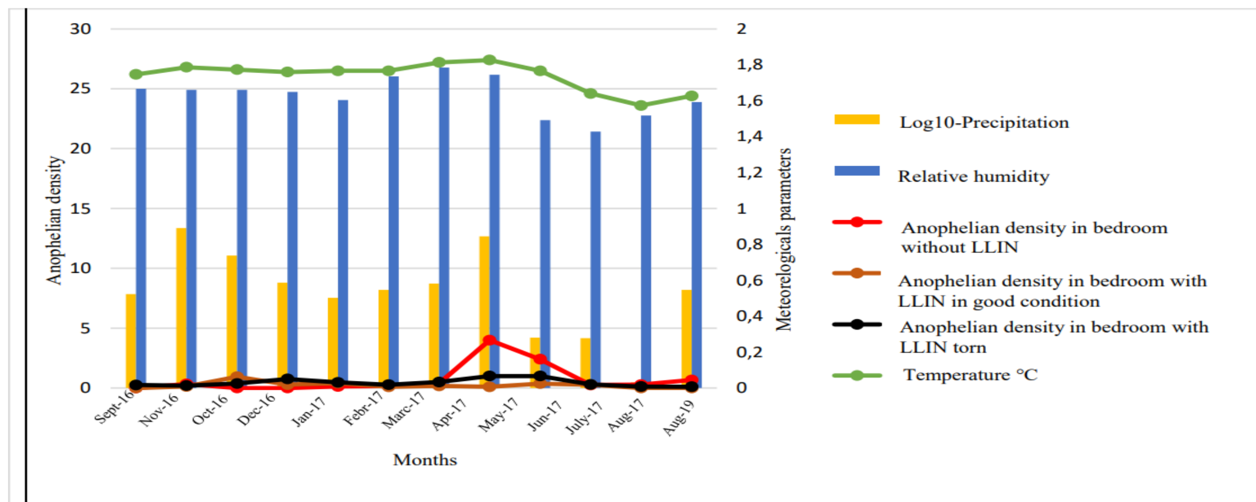

Figure 3. Monthly variation of *Anopheles gambiae s.l.* densities according to the absence or presence of LLINs in good condition or torn in Kintele between September 2016 and August 2017.

In bedrooms without LLIN, anopheline densities increased slightly from September to March, reaching a peak in April. Subsequently, they remained relatively constant between June and August.

For bedrooms with torn LLINs, anopheline densities showed a consistent pattern from September to August.

Similarly, in bedrooms with LLINs in good condition, anopheline densities were relatively constant from September to August.

Statistical tests revealed no significant differences in anopheline densities based on collection month (Kruskal-Wallis test; p-value = 0.348) or bedroom type (Kruskal-Wallis test; p-value = 0.1821). Additionally, the Kruskal-Wallis test indicated that anopheline densities in bedrooms without LLINs and those with torn LLINs were statistically identical (Kruskal-Wallis test; p-value = 0.063).

In bedrooms without LLINs, anopheline densities slightly increased from September to October, followed by a decrease from November to December (Figure 4). Densities then increased from December to February, peaking in February. A significant decrease in density was observed in June before a gradual increase

through July to August 2019.

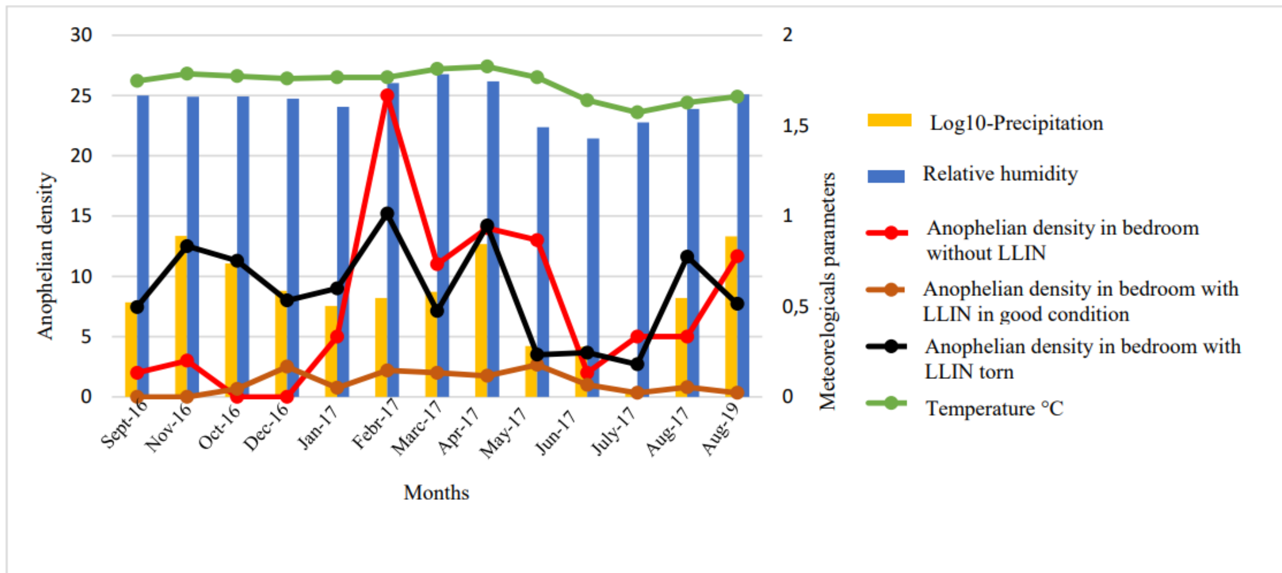

Figure 4. Monthly variation of *Anopheles gambiae s.l.* densities based on the absence or presence of LLINs in good condition or torn between September 2016 and August 2017 and in August 2019 in Djoumouna.

For bedrooms with torn LLINs, four peaks in anopheline densities were observed in October, February, April, and August. Densities then slightly decreased in August 2019.

In bedrooms with LLINs in good condition, densities remained relatively stable from September to June, showing minimal variation throughout the study period. Thus, no distinct peak was observed. Densities then declined from June to August, nearly reaching zero.

Statistical tests indicated that there was no significant difference between in anopheline densities based on collection months (Kruskal-Wallis test; p-value = 0.1213). However, the Kruskal-Wallis test revealed a significant difference in anopheline densities based on bedroom type (Kruskal-Wallis test; p-value = 0.0003).

3.2. Identification of Species of the *Anopheles gambiae s.l.* Complex

A total of 343 females belonging to the *An. gambiae* complex was identified by PCR in the two study localities. Molecular analysis of the sampled females reaveled that the *An. gambiae s.l.* complex consisted of *An. gambiae* (72.3%), *An. coluzzii* (27.1%), *An. arabiensis* (0.3%) and hydrids (between *An. gambiae and An. Coluzzii*) (0.3%) in both locations. The Kruskal-Wallis statistical test showed that there was a significant difference in the species collected between the two localities (Kruskal-Wallis; p-value = 0.0426). Furthermore, a significant difference was observed in the species composition of the *Anopheles gambiae s.l.* complex in the two localities.

The seasonal distribution of *Anopheles gambiae s.l.* complex in Kintele and Djoumouna is outlined in Table 2.

Table 2. PCR identification of *Anopheles gambiae s.l.* complex species collected during this study.

Species	Kintele		Djoumouna		Total	
	number	%	number	%	number	%
An. gambiae	56	81.2	192	70.1	248	72.3
An. coluzzii	12	17.4	81	29.6	93	27.1
An. arabiensis	1	1.4	0	0	1	0.3
Hybrid (*An. gambiae*/*An. coluzzii*)	0	0	1	0.4	1	0.3
Total	**69**	**100**	**274**	**100**	**343**	**100**

Table 3. Identified species of the *Anopheles gambiae s.l.* complex.

Season	Months	Kintele			Djoumouna			Number of *An. gambiae s.l.* sampled	Total number *An. gambiae s.l.* collected
		An. gambiae	*An. coluzzii*	*An. arabiensis*	*An. gambiae*	*An. coluzzii*	hybrid		
Rainly	Oct.-Nov. 2016	8	1	0	48	17	0	74	206
	Dec. 2016-Jan. 2017	0	0	0	26	16	0	42	178
	Feb.-Mar. 2017	8	9	0	7	7	0	31	204
	Apr.-May 2017	21	2	1	29	1	0	54	184
Dry	Jun.-Jul. 2016	11	0	0	21	19	0	51	88
	Aug. (2017, 2019)-Sept. 2016	8	0	0	61	21	1	91	270
Total		56	12	1	192	81	1	343	1130

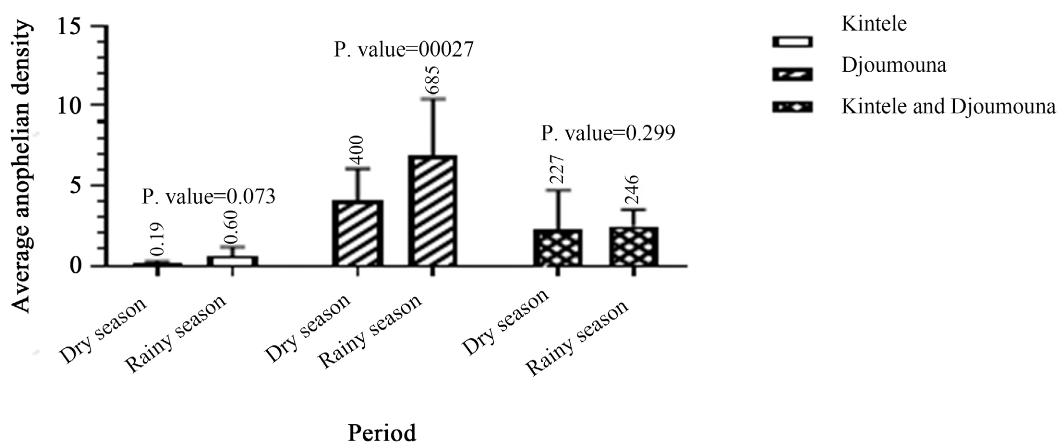

Figure 5. Monthly distribution of *Anopheles gambiae s.l.* based on collection seasons in Kintele and Djoumouna between September 2016, August 2017 and in August 2019 in Djoumouna alone.

Table 3 and Figure 5 present the species within the *An. gambiae s.l.* complex identified by season. All three species were collected during the rainy season.

The KHI-2 statistical test revealed a significant difference in the densities of *An. gambiae* and *An. coluzzii* between Kintele and Djoumouna during the rainy season. The KHI-2 statistical test results for each species were as follows: (KHI-2 = 26.10; p-value < 0.001) for *An. gambiae* and (KHI-2 = 21.32; p-value < 0.001) for *An. coluzzii*. Similarly, in the dry season, the test demonstrated a significant difference in the densities of *An. gambiae* and *An. coluzzii* between Kintele and Djoumouna (KHI-2 = 32.87; p-value < 0.001).

In Kintele, during the rainy season, the monthly distribution of *An. gambiae* species was identified using the Kruskal-Wallis test (p-value = 0.0058). *An. gambiae* was the only species identified during the dry season. Both *An. gambiae* and *An. coluzzii* exhibited significantly higher abundance during the rainy season compared to the dry season (Kruskal-Wallis test; p-value = 0.0477).

In Djoumouna *An. gambiae* exhibited significantly greater prominence than other species during both the rainy season (Kruskal-Wallis test; p-value = 0.0006) and the dry season (Kruskal-Wallis test; p-value = 0.0417).

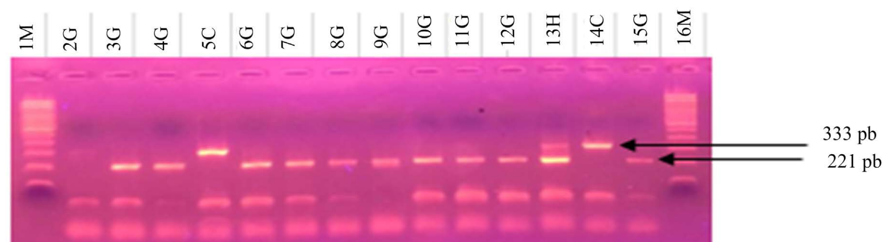

Figure 6. Diagnostic gel for species identification of the *An. gambiae s.l.* complex.

Caption:

1) Wells 1 and 16: 1 kb base-pair ladder.
2) Wells 2, 5 and 14 : Individuals *An. coluzzii* (C).
3) Wells 3, 4, 6, 7, 8 to 12 and 15: Individuals *An. gambiae* (G).
4) Well 13: *An. gambiae/ An. coluzzii* hybrid individual (H).

Figure 6 shows an agarose gel showing separated DNA bands. Within this gel, two species were identified, and the most identified species was *An. gambiae*, comprising 10 out of the 14 identified females. In the case of *An. coluzzii*, 3 females out of 14 specimens were identified. Additionally, one hybrid female was identified.

The assessment of the entomological indices and transmission evaluation gives the following results:

Aggressive density represents the average number of bites an individual receives in the specified area within a unit of time.

Parity rate indicates the proportion of parous females (those having already laid eggs) within a sample of anopheline population.

The sporozoite index is the proportion of females observed with sporozoites in their salivary glands among the dissected females.

The entomological inoculation rate expresses the number of infective bites an individual experiences within a specific time frame. It is a measure of the intensity of malaria transmission within a given area.

These results show that the highest aggressive density (0.22 p/h/n) was recorded in bedrooms without LLINs, while the lowest (0.07 p/h/n) was observed in bedrooms with LLIN in good condition.

1) Regarding the number of infected anopheles, 2 females were collected from bedrooms with LLINs in good condition, in contrast to 1 female from bedrooms without LLIN. Notably, no infective females were collected from bedrooms with torn LLINs.

2) The sporozoite index (SI) exhibited a higher greater value (4.17%) in bedrooms with LLINs in good condition, while it was null in bedrooms with torn LLINs.

3) Concerning the rate of parity, bedrooms with torn LLINs showed a higher rate (38.09%), while bedrooms with LLINs in good condition displayed a lower rate (28.7%).

4) The entomological inoculation rate indicated one infective mosquito bite per person every 5.5 months (166.6 days) in bedrooms without LLINs, and one infective bite per person every 11.1 months (333 days) in bedrooms with LLIN in good condition.

Statistical tests showed significant difference in aggressive rates across thedifferent bedroom types (KHI-2 = 24.94; p-value < 0.0001). Notably, the aggressive rates in bedrooms without LLINs and with torn LLINs were significantly different (KHI-2 = 8.55; p-value = 0.0034). While there was no significant difference in the number of infective females and sporozoite indices between bedroom types (Kruskal-Wallis test; p-value = 0.9723), the same was true when comparing bedrooms without LLINs and with torn LLINs (Fisher's exact test; p-value = 0.8541). Additionally, there was no noteworthy difference in parity rates when comparing the various bedroom types of (KHI-2 = 2.53; p-value = 0.2823). Likewise, the party rates in bedrooms without LLINs and with torn LLINs were identical (KHI-2 = 0.21; p-value = 0.6501). Furthermore, disparities in entomological inoculation rates were observed among different bedroom types (KHI-2 = 77.34; p-value < 0.0001). Lastly, significant differences in entomological inoculation rates were noted between bedrooms without LLINs and with torn LLINs were significantly different (KHI-2 = 101.14; p-value < 0.0001).

Table 4 presents the entomological indices of malaria transmission over the entire study period in Djoumouna.

These findings reveal that the most substantial aggressive density (1.73 p/h/n) was recorded in bedrooms lacking LLINs. In contrast, the lowest density (0.13 p/h/n) was recorded in bedrooms with LLINs in good condition.

1) Regarding the number of infected anopheles, the highest number (15 fe-

males) was obtained from bedrooms with torn LLINs. Conversely, the lowest number (1 female) was found in rooms with LLINs in good condition.

2) Regarding the sporozoite index (SI), it was higher (2.14%) in bedrooms without LLINs and in bedrooms with LLINs in good condition. The lowest index (1.86%) was recorded in bedrooms with torn LLINs.

3) Regarding the rate of parity, the highest rate (82.23%) was observed in bedrooms with torn LLINs, while the lowest rate (4.39%) was found in bedrooms with LLINs in good condition.

4) Regarding the entomological inoculation rate, it was estimated to one infective mosquito bite per person every 27 days in bedrooms without LLINs. In bedrooms with LLINs in good condition, there was one infecting bite per person every 1.2 years (370.3 days). For bedrooms with torn LLINs, the rate was one infective bite per person every 2.2 months (66.6 days).

Table 4. Entomological indices by type of bedroom in Djoumouna.

LLIN Status	Number of persons	Aggressive density	Number of dissected Culicidae	Infected Culicidae	Sporozoite index (SI) (%)	Number of pares	Parturiency rate (%)	Entomological inoculation rate	*i.e.* one infecting bite every
LLIN without	75	1.73 P/h/n	140	3	2.14%	79	13. 37%	0.037	27 days
LLIN in good condition	227	0.13 P/h/n	48	1	2.08%	26	4.39%	0.0027	370.3 days or 1.2 years
LLIN torn	928	0.81 P/h/n	806	15	1.86%	486	82.23%	0.015	66.6 days or 2.2 months
Total	1230	0.74 P/h/n	994	19	1.91%	591	100%	0.014	71.4 days or 2.3 months

Statistical tests show significant differences in aggressive rates among the various bedroom types (KHI-2 = 431.78; p-value < 0.0001). The aggressive rates in bedrooms without LLINs and with torn LLINs were significantly different (KHI-2 = 17.25; p-value < 0.0001). However, there was no significant difference in the number of infective females and sporozoite indices among the different types of bedroom types (Kruskal-Wallis test; p-value = 0.9712). Likewise, no significant difference was observed between bedrooms without LLINs and those with torn LLINs (Fisher's exact test; p-value = 0.7107). Similarly, there was no significant difference in parity rates across the different bedroom types (KHI-2 = 1.33; p-value = 0.5152). Furthermore, the parity rates in bedrooms without LLINs and with torns LLIN were similar (KHI-2 = 0.74; p-value = 3889). Lastly, a significant difference in entomological inoculation rates between the different bedroom types (KHI-2 = 140.37; p-value < 0.0001) was noted.

4. Discussion

The results of this study underscore the essential role of LLINs in people's efforts to protect themselves against malaria and culicidal nuisances. However, it's worth noting that a significant number of investigated bedrooms (76 in total) lacked LLINs. Financial constraints posed the primary hindrance to obtaining new LLINs. Indeed, ensuring a family's protection often requires more than two LLINs, a challenge particularly for families with precarious incomes.

Concerning the anopheline fauna, only *An. gambiae s.l.* and *An. funestus* species were collected. The absence of the other species described in Congo [6] [14] [16] [22]-[24] could be attributed to their sensitivity to insecticides, which might have contributed to their decline. The species within the *An. gambiae s.l.* complex predominated in the collection. This prevalence is due to their ecological plasticity to various environmental conditions and their swift developmental cycle [25]. This ecological plasticity enables them to thrive in diverse settings for their growth. According to the bedroom types, the highest densities were found in bedrooms with torn LLINs (**Table 1**). These higher densities are linked to the presence of holes on these LLINs, compromising their physical barrier. As a result, these LLINs have reduced efficacy, including the loss of their chemical barrier. This decrease in efficiency facilitated anopheles mosquitoes' entry into theserooms and their access to sleepers. In contrast, the lowest densities were recorded in bedrooms with LLINs in good condition (**Table 2**). The intact chemical and physical barriers of these LLINs prevented mosquitoes from entering the rooms and feeding on the sleepers. When considering location and season, the highest anopheline densities were noted in the village of Djoumouna during the rainy season (**Figure 4**). This abundance can be attributed to the diverse larval sites within the village, such as temporary sites that are filled with rainwater during the wet season and fish ponds that become permanent sites when they're emptied or obstructed.

It is important to highlight that Kintele experienced increased urbanization, leading to heightened land pressure. This diminished available space for larval breeding sites, resulting in reduced larval production. Moreover, sandy soils' characteristics in Kintela promote rapid rainwater infiltration, as well as runoff and evaporation, which collectively lead to the rapid drying of temporary breeding sites 5 days after the rain. This subsequently redues adults mosquito densities. Molecular analysis of the *An. gambiae s.l.* complex identified three species in the two study localities (**Table 2**). The predominant species were *An. gambiae* and *An. coluzzii*, coexisting in sympatry. Their distribution aligns with their geographical range [26]. In Congo, these two species were initially observed in 2009 in the village of Boutoto [27]. *Anopheles gambiae* predominantly identified in this study, matches findings from Koekemoer *et al.* who identified it as the primary collected species (old molecular form S) [27]. *An. gambiae* tends to dominate due to its high ecological plasticity and ability to colonize a wide variety of larval habitats, including temporary rain-dependent habitats, which, combined with faster larval development, favors its relative predominance. *An. coluzzii*, on the other

hand, is more frequently associated with semi-permanent or permanent anthropogenic sites such as irrigated rice fields, ponds, and pools, a pattern observed in several West African contexts. These ecological differences temporary habitats for *An. gambiae* versus semi-permanent/permanent habitats for *An. coluzzii* have been confirmed by recent work in West Africa and are consistent with the observation of fish ponds as favorable breeding sites for *An. coluzzii* in locations such as Djoumouna [28] [29]. *An. coluzzii,* the second most collected species, was primarily found in Djoumouna, where the fish ponds potentially serve as preferred breeding sites. Studies elsewhere have revealed ecological disparities between *An. gambiae* and *An. coluzzii*. The latter species tends to favor semi-permanent and permanent sites associated with year-round human activities [28]. Notably, such sites are absent in Kintele. In the low densities of *An. coluzzii* in Djoumouna may be due to predation and competition with other aquatic species within fish ponds, particularly during the larval stages. Lastly, *An. arabiensis*, a species adapted to savannahs and sparse forests with a tolerance for dry climates, was sparsely represented, with only one female collected [26] [30] [31].

In Congo, changes observed in *An. arabiensis* distribution suggest its adaptation to the humid tropical climate, likely driven by ongoing climate change. A 2017 study on vector distribution supports this claim, indicating that climate change facilitates the expansion of this species, which, in turn, impacts the distribution of mosquitoes and the epidemiology of vector-borne diseases [26]. The discovery of only one hybrid (*An. gambiae*/*An. coluzzii*) (0.3 %) implies limited gene flow between *An. gambiae* and *An. coluzzii*. This suggests that they are evolving along distinct evolutionary paths. This observation is in agreement with previous studies in Central Africa, which have consistently reported minimal gene flow between these two species and hybrid frequencies lower than 1% [32].

The physiological status of the collected females revealed that blood-fed and semi-gravid females were the most abundant. This suggests an endophilic behavior among anopheline populations in these areas.

The average rate of aggression, analyzed according to room type and location, was highest in rooms without long-lasting insecticide-treated mosquito nets (LLINs). This indicates that people sleeping in these rooms are more exposed to mosquito bites due to the lack of protective measures. In contrast, the lowest rates were observed in rooms equipped with LLINs in good condition in both locations studied: the combined physical and chemical barrier limits the access of *Anopheles* mosquitoes to the sleeper and encourages them to leave the room prematurely. These results are consistent with recent studies showing a significant decrease in aggressiveness and vector density following the use of treated nets: for example, a multicenter trial in West Africa observed a significant reduction in bites per person per night thanks to LLINs with enhanced active ingredient (%) [33]. Similarly, a field survey in Tanzania estimated that more than 75% of infectious exposures could be prevented by complete and effective LLIN coverage [34]. Parity rates exceeding 80% in Djoumouna's bedrooms with torn LLINs indicate that, despite

high LLIN coverage, they do not significantly reduce the longevity of females reaching the critical epidemiological age. Consequently, LLINs may not substantially decrease the vectorial capacity of *Anopheles* mosquitoes.

The results obtained with the sporozoite index indicate that species within the *Anopheles gambiae s.l.* complex are the primary contributors to malaria transmission. However, it does not differentiate between species carrying sporozoites. Regarding the entomological inoculation rate, malaria transmission in Djoumouna's bedrooms without LLINs amounted to one infective bite per person every 27 days. This is likely due to the absence of LLINs barriers, the proliferation of anopheles, and the presence of gametocyte carriers.

However, the sporozoite index observed in Kintele appears to be higher in bedrooms with LLINs in good condition (4.17%) than in those without LLINs (2.56%), a result that is counterintuitive at first glance. This observation is nevertheless based on a very small number of infected mosquitoes, with only two infected females collected in rooms with intact LINs compared to one in rooms without LLINs, and no infected females in rooms equipped with torn LLINs. This small sample size severely limits the interpretative scope of these results and does not allow us to conclude that there is a real increase in the risk of transmission associated with the use of LLINs.

It is therefore likely that this difference in the sporozoite index is related to a sampling effect rather than a real biological or behavioral phenomenon. Furthermore, the low overall vector density in Kintélé could lead to a dilution of the risk of transmission, due to the relatively high number of human hosts compared to the number of *Anopheles* mosquitoes, thus contributing to the low intensity of transmission observed despite the occasional detection of infected mosquitoes.

These results demonstrate that the use of long-lasting insecticide-treated nets (LLINs) in good condition significantly reduces the entomological inoculation rate (EIR) of malaria in communities that use them. In Djoumouna and surrounding areas, the widespread and consistent use of LLINs by the population has led to a marked decline in malaria transmission intensity. However, the effectiveness of this preventive measure remains challenged by local ecological conditions that are highly favorable to vector development, particularly the presence of permanent fish ponds. These ponds constitute stable and highly productive breeding sites, supporting the continuous proliferation of mosquito vectors belonging to the *Anopheles gambiae s.l.* complex, notably *An. gambiae*, *An. coluzzii*, and *An. arabiensis*, as well as hybrid forms. Despite these constraints, malaria transmission has declined substantially compared to historical levels, dropping from an average of approximately 1000 infective bites per person per year in the 1970s, highlighting the significant impact of vector control interventions such as LLINs [35] to 14 ib/p/year during this study. Similar reductions have been observed in other regions, such as Burkina Faso, where entomological inoculation rates decreased from 55 ib/p/year to 3 ib/p/year after the introduction of deltamethrin-treated nets [36]. However, it is important to note that the use of torn LLINs, distributed

five years prior to the study, is no longer effective, as the average duration of efficacy is approximately three years. This reduction in effectiveness can be exacerbated by maintenance practices (e.g., washing with detergent) and the misuse of these LLINs, leading to tears [37].

5. Conclusions

This study confirms that *Anopheles* mosquitoes of the *Anopheles gambiae s.l.* complex are the main vectors of malaria in the areas studied, with the identification of three major species (*An. gambiae*, *An. coluzzii*, and *An. arabiensis*), as well as hybrid forms. The specific composition and distribution of *Anopheles* mosquitoes show clear heterogeneity between the two locations, reflecting distinct ecological contexts. In Djoumouna, a rural area, the presence of permanent fish ponds promotes higher vector densities, contributing to more intense malaria transmission than in Kintélé, an urban area, where *Anopheles* densities are lower. These differences in the composition and abundance of vector species highlight the importance of accurate identification of *Anopheles* mosquitoes in order to guide vector control strategies adapted to local specificities, in particular by integrating larval habitat management in rural areas.

In addition, long-lasting insecticide-treated mosquito nets (LLINs) appear to be an effective public health tool for reducing malaria transmission in both urban and rural areas. However, their effectiveness depends heavily on their physical condition and correct use. Therefore, keeping LLINs in good condition, combined with ongoing public awareness campaigns, remains an essential condition for optimizing their impact and keeping malaria transmission as low as possible, in addition to targeted control strategies based on the specific composition of vectors in each ecological context.

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Conflicts of Interest

The authors declare no conflicts of interest.

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