

Original Research Articles

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF ANOPHELES MOSQUITOES AND THEIR BEHAVIOURAL PATTERNS IN UYO, NIGERIA

ABSTRACT

Aims: Malaria is a public health problem in Nigeria. Knowledge of some baseline data such as Human Biting Rates (HBR) is needed to assess the epidemiology of the disease and in estimating the vector-human contact. This study provides information on the behavioural patterns of anopheles mosquitoes in Uyo.

Study design: A six months study was carried out in Uyo, Nigeria where no information exists on the major malaria vectors associated with human malaria. Sample collection was carried out between May and October 2013 using Knockdown and Human Landing Catches (HLC) techniques.

Methodology: Adults mosquitoes were collected in two areas in Ewet Offot Community. Nine houses were randomly selected in each location using Simple Random Sampling Method. Mosquitoes belonging to the *Anopheles gambiae* complex were further characterized and identified by a molecular method using Polymerase Chain Reaction (PCR).

Results: Two anophelines species were collected by the sampling methods consisting of 21(23.3%) *Anopheles nili* and 69(76.7%) *Anopheles gambiae* complex. A Polymerase Chain Reaction (PCR) based test on the *An. gambiae* complex identified 66(96.0%) as *An. gambiae sensu stricto*. The study also revealed that the resting behaviour of *An. gambiae* complex species in this area is endophilic whereas the resting behaviour of *An. nili* is exophagic/exophilic. The peak biting activity of *An. gambiae* complex species occurred at 2300 hours (indoor) and 1900 hours (outdoor) in July whereas that of *An. nili* occurred at 2200 hours (indoor) and 1800 hours (outdoor) in June. The Human Biting Rates (HBR) recorded for *An. gambiae* was higher than *An. nili* and the total number *An. gambiae* collected was more than *An. nili*.

Conclusion: There is a need for a comprehensive knowledge on the behaviour and heterogeneities that exist within and among malaria vector species in this area if the goal of malaria elimination is to be achieved.

Keywords: *An. gambiae*, *An.nili*, PCR, Nigeria

1. INTRODUCTION

Malaria is a vector-borne disease caused by single celled parasites of the genus *Plasmodium*. It is transmitted by infected female anopheles mosquitoes [19]. It is the major public health problem in Nigeria contributing a quarter of the malaria burden in Africa [20] The disease is the most important cause of human morbidity and mortality and with enormous medical, emotional and economic impact in the world [3]. The Federal Ministry of Health [6] estimates that approximately 30% of the Nigerian population lives in areas of high to very high malaria transmission intensity while 67% live in moderate transmission zone. Also, the disease is responsible for 60% outpatient visits to health facilities, 30% childhood mortality and 11% maternal mortality thereby imposing great burden on the country in terms of pains and trauma suffered by its victims [6].

Malaria is caused by four different protozoa in the *Plasmodium* genus and these are: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium falciparum*, the most dangerous of the four. The zoonotic species, *Plasmodium knowlesi* can also cause severe infections in humans [10]. Over 90% of the population in Nigeria is at risk of acquiring malaria infection because of drug resistance in the parasite strain, insecticide resistance in the vectors and the presence of very efficient mosquito vectors of *Plasmodium falciparum* [11]. Vectorial capacity takes into account environment, behavioural, cellular and biochemical factors that influence the association between vector, pathogen transmitted by the vector and the vertebrate host to which the pathogen is transmitted [1].

The burden of malaria remains high in many African countries, despite increasing effort to control the disease due to a persistent high level of transmission [8]. Malaria transmission is variable from one area to another and this has an impact on its epidemiology and control [3]. The development of the malaria parasites inside the mosquito (sporogonic cycle) is more rapid as the temperature rises and increased

rainfall and stagnant pools of water or surface water provide hospitable breeding grounds for the mosquitoes [4].

The role of anophelines in transmitting the disease depends on their occurrence and preference to feed on humans [8] which in turn is affected by socio-economic as well as environmental factors [8]. Thus, preventing humans from the bite of vectors can reduce malaria transmission. However, implementation of preventing tools requires knowledge on occurrence, feeding behaviour of the vectors [15].

In Nigeria, there is little baseline information on vector populations and variation in the intensity of malaria transmission but the use of vector control methods via Indoor Residual Sprays (IRS) and Insecticide Treated Nets (ITNs) are applied without regard to the local epidemiology of the disease especially the relationship to vector behaviour and intensity of transmission [11].

Uyo is the capital of Akwa Ibom State and it is an ecological area that has undergone serious environmental modifications over the years owing to rapid growth in population and urbanization. Such modifications can lead to ecological changes that can affect vector population structure, distribution and density, all which would impact on the efficiency in transmitting malaria. Therefore, this study seeks to describe the behavioural patterns of anopheline mosquitoes in Uyo.

2. METHODOLOGY

2.1 Study Area

The study was conducted in Ewet Offot in Uyo Local Government area. Uyo lies between latitude 5.05o North and Longitude 80o East. It is within the equatorial rainforest belt, which is a tropical zone that houses vegetation of green foliage of trees, shrubs and oil palm trees. Local climate is tropical, with dry season between November and March and wet season between April and October.

The average temperature ranges from 23oC -31oC, providing an ideal climate for malaria transmission throughout the year. Ewet Offot is located along Nwaniba road and beside Oron road in Uyo Local Government Area. Based on their dense population 2 areas were selected for the study. These areas were Orok Close and Urua Udofia Streets which are 2 kilometers apart from each other. These areas are usually characterized by stagnant pools in the rainy season and Urua Udofia Streets is surrounded by streams, valleys and borrow pits. Most houses were built in clusters in Urua Udofia Streets and some compounds consist of a group of houses separated from each other by gardens in Orok Close.

2.3 Sampling Methods

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Adult mosquitoes were collected in two areas in Ewet Offot Community (Orok Close and Urua Udofia Streets). Nine houses were randomly selected in each location using Simple Random Sampling Method. Sample collection was carried out for six months (May- October, 2013) using the following techniques:

2.3.1 Knockdown Technique

This technique involved the laying of Calico cloth on the floor and on the surfaces of immovable household furniture. The houses were sprayed using knockdown Insecticide consisting of Transfluthrin 0.25% and Permethrin 0.5% according to the manufacturer's instruction. After 10 minutes the Calico cloth was carefully taken out of the house and knocked-down mosquitoes were collected using forceps. Three houses with nets and three houses without nets were used for the collection of samples using this technique.

2.3.2 Human Landing Catches (HLC)

Human Landing Catches (HLC) was carried out according to World Health Organization standard procedure [18] which adopts the standard human bait collector method. This technique was performed two times a month 6pm -10pm outdoor and 10pm- 6pm indoor in three compounds different from the one that was used for knockdown technique. These compounds were used for the collection of mosquitoes using this technique throughout the study period.

2.4 Sample Preservation

117 All the adult mosquitoes that were collected were kept in a labeled 1.5ml eppendorf tubes containing
118 **silica gel desiccant**. Samples were stored in the laboratory at room temperature (350C-370C).
119

120 2.5 Identification of Samples

121 2.5.1 Morphological Identification

122 Anopheles mosquitoes were morphologically identified and differentiated from *Culex quinquefasciatus*
123 and *Aedes aegypti* using Binocular microscope with the aid of identification Manual [14,2] in a WHO
124 Reference Laboratory.
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126 2.5.2 Molecular Identification of Anopheles Mosquitoes

127 Polymerase Chain Reaction (PCR) technique of Scott, Brogon and Collins [12] and minor modifications
128 as detailed in [17] was used to identify species of *Anopheles gambiae*.

129 The steps used in carrying out PCR analysis were:

- 130 i. Extraction of DNA
- 131 ii. Amplification of DNA
- 132 iii. Gel Electrophoresis

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134 The DNA of *Anopheles gambiae* complex were extracted from the legs and wings and were conserved
135 dry in the freezer then amplified in enzymatic PCR of gene [17].

136 *Anopheles gambiae* primers used were as follows:

- 137 GA (Gambiae)
- 138 AA (Arabiensis)
- 139 ME (Melas)
- 140 QD (Quadriannulatus)
- 141 UN (Universal)

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143 **Procedure:** *Anopheles gambiae* primers were measured according to their correct proportion and mixed
144 properly. 12.5µl of PCR buffer was added to it. Master Mix was measured to each eppendorf tubes and
145 1µl of DNA was added to each tube. Each tube was loaded in the PCR machine and PCR program was
146 chosen. Following this, 1.5% agarose gel with TBE buffer and 3.0µl of Ethidium bromide was prepared. At
147 this point, 12.5µl PCR product was loaded with 1µl loading buffer into each well. Finally, 10µl of standard
148 marker was loaded per gel. PCR product ran at 100 volts. Gel documentation photograph was taken
149 using Syngene bio-imaging system.

150 The three processes involved in PCR analysis were:

- 151 i. Denaturation: DNA strand was separated and it occurred at 94°C (for 30 seconds)
- 152 ii. Annealation: Binds the primer to the specific DNA (occurred at 500°C-540°C).
- 153 iii. Elongation or Extension: Binds to the primer and Deoxynucleotide triphosphate (dNTPs) from the
154 DNA polymerase synthesizes a new strand (occurred at 72°C).

155 These processes were programmed inside an automated PCR machine.

156 The PCR DNA enabled the identification of sibling species as *Anopheles gambiae sensu stricto (s.s)*,
157 *Anopheles arabiensis*, and *Anopheles melas* by comparison with a molecular weight markers.
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159 2.6 Statistical Analysis

160 The entomological parameters that were determined was the Human-Biting Rates (HBR), calculated as
161 the number of mosquito bites received per person per night of collection using the formula [9].
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163 The rate of endophagy, defined as the proportion of mosquitoes caught indoors by HLC over the total
164 numbers of mosquitoes collected by HLC (indoors and outdoors).

165 Chi-square test was used to compare the results from the two locations.
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168 3. RESULTS

170 3.2 Morphological Identification of Mosquitoes

A total of 1300 mosquitoes were collected from two areas in Ewet Offot within the period of 6 months but only 700 were used for morphological identification and 90 (12.8%) of these were identified as female anopheles mosquitoes consisting of *An. nili* 21 (23.3%) and *An. gambiae* complex 69 (76.7%) (Table 3). *Culex quinquefasciatus* were 300 (42.9%) in Orok Close and 214 (30.6%) in Urua Udofia Streets. *Aedes aegypti* collected in Orok Close were 46 (6.6%) and 50 (7.1%) in Urua Udofia Streets (Table 2).

Aedes aegypti and *Culex quinquefasciatus* collected were excluded from the study because they are not malaria vectors. PCR analysis was not used to identify the specific sibling species within the *Anopheles nili* complex. During the rainy season however, when mosquito densities are high, mosquito nets and fumigating coils were frequently used for personal protection. Household ownership of mosquito nets was around 36% in Orok Close and 25% in Urua Udofia Streets.

3.3 Molecular Characterization of Anopheles Mosquitoes

The *Anopheles gambiae* species specific PCR was employed to distinguish the species present within the *An. gambiae* of the total *An. gambiae* collected 66 (96%) were identified as *An. gambiae* s.s and 3 (4%) were negative (Figure 1). DNA bands were visualized under short wave length UV light. The PCR DNA (Figure 1) enabled the identification of sibling species of *An. gambiae* complex as *An. gambiae* s. s by comparison with a DNA ladder (a molecular weight marker).

Table 1: Classification into species based on morphological characteristics

Features	<i>An. gambiae</i>	<i>An. nili</i>
Wings	dark spots on the wing veins	pale wings
Proboscis/ Palps	black spot on the palps	white spots on the palps
Legs	spotted or speckled legs	dark legs
Colour	yellowish brown to brown	bright yellowish red

Table 2: Species composition of female mosquitoes collected in both Orok Close and Urua Udofia Streets.

Areas	Mosquito species	No. identified	%
Orok close	<i>An. gambiae</i> complex	20	2.9
	<i>An. nili</i> complex	7	1.0
	<i>Culex quinquefasciatus</i>	300	42.9
	<i>Aedes aegypti</i>	46	6.6
Urua Udofia Streets	<i>An. gambiae</i> complex	49	7.0
	<i>An. nili</i> complex	14	2.0
	<i>Culex quinquefasciatus</i>	214	30.6
	<i>Aedes aegypti</i>	50	7.1

Table 3: Sibling species composition of the two anopheles vector groups identified in Orok Close and Urua Udofia Streets.

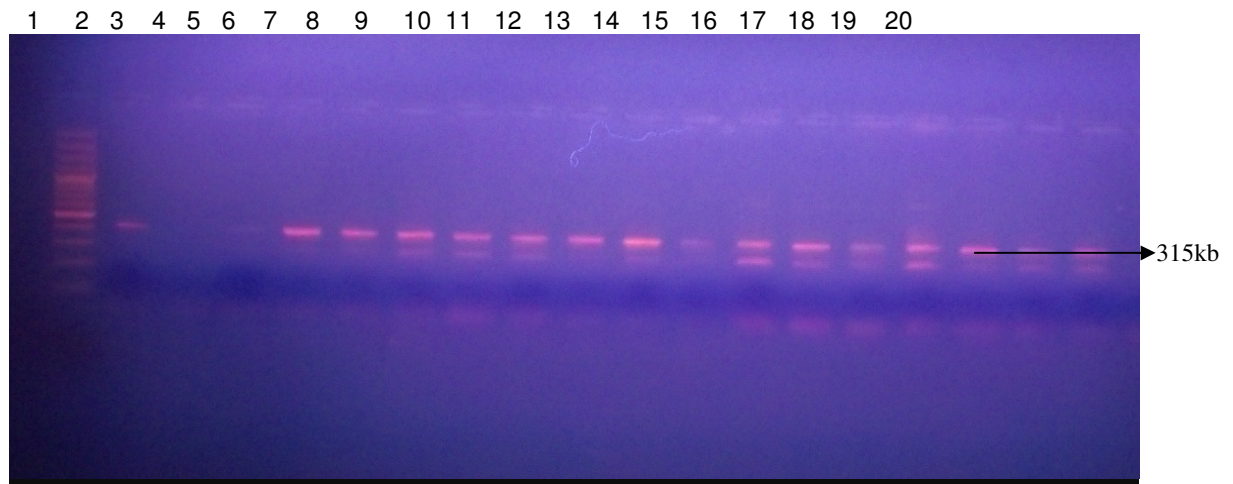
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Location	Group	Sibling species	No. identified	%
Orok Close	<i>An. Gambiae</i>	<i>An. gambiae</i> s.s	20	22.2
	<i>An. Nili</i>	-	7	7.7
Urua Streets	<i>An. Gambiae</i>	<i>An. gambiae</i> s.s	49	54.4
	<i>An. Nili</i>	-	14	15.6
Total			90	100

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Fig I: Molecular Characterization of sibling species of *An. gambiae* complex

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Lane 1 = 100kb DNA ladder, Lane 2 = Standard strain of *An. gambiae* s. s
Lanes 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, 20 = *An. gambiae* s. s
The molecular weight of *An. gambiae* s. s is 315kb.

Anopheles gambiae and *Anopheles nili* displayed similar biting activities. The observed differences in the proportion of *Anopheles gambiae* collected showed that the total number of *Anopheles gambiae sensu stricto* collected outdoor and in houses without nets was more than those collected indoor and with nets (Table 4 and Table 5). Biting generally commences at 1800 hours (60% of samples were collected outdoors). *Anopheles gambiae* indoors peaked at 2300 hours and 0300 hours (Table 6 and Table 7).

Anopheles nili were collected resting outdoors and few were found resting indoor after feeding. No male sample of any anopheles mosquito was collected by Human Landing Catches (HLC). However, there was statistical significant difference between indoor and outdoor host seeking behaviour of *An. gambiae* and *An. nili* in the area.

The population of the species collected in the two areas shown on Tables 4 and 5 showed that the proportion of *An. gambiae* and *An. nili* complex collected using HLC outdoor was more than *An. nili* in houses without nets in both Orok Close and Urua Udofia Street. The highest number of *An. gambiae* s.s was collected and recorded in the July after two months onset of rain. The highest number of *An. nili* complex was recorded in the June Figure 2.

However, the total number of *An. gambiae* collected across the two areas was more than the number of *An. nili* caught within the same period. The Human Biting Rates (HBR) recorded for *An. gambiae* s.s was higher than *An. nili* complex although increase in man biting rate in each species coincides with the level of rainfall reaching maximum at different months throughout the study period Table 8. The rate of endophagy of *An. gambiae* s.s in Orok close was 0.14 and *An. nili* was 0. In Urua Udofia the rate of endophagy of *An. gambiae* was 0.3 and *An. nili* was 0.2.

241 **Table 4:** Species composition of indoor and outdoor biting *An. gambiae* and *An. nili*
 242 mosquitoes in the two different areas in Ewet Offot using human landing catches
 243 (HLC) method.
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Areas	Indoor		Outdoor	
	<i>An. gambiae</i> No. (%)	<i>An. nili</i> No. (%)	<i>An. gambiae</i> No. (%)	<i>An. nili</i> No. (%)
Orok Close	1 (20)	0	6 (33.3)	2 (20)
Urua Udofia	4 (80)	2 (100)	12 (66.7)	8 (80)
Streets				
Total	5 (100)	2 (100)	18 (100)	10 (100)

246 **Table 5:** Total number of *An. gambiae* and *An. nili* mosquitoes collected in houses with nets
 247 and without nets using knock-down technique in two different areas in Ewet Offot Community.
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Areas	Houses with nets		Houses without nets	
	<i>An. gambiae</i> No. (%)	<i>An. nili</i> No. (%)	<i>An. gambiae</i> No. (%)	<i>An. nili</i> No. (%)
Orok Close	2 (25)	1 (50)	10 (26.4)	2 (28.6)
Urua Udofia	6 (75)	1 (50)	28 (73.6)	5 (71.4)
Streets				
Total	8 (100)	2 (100)	38 (100)	7 (100)

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253 **Table 6:** Indoor host seeking behaviour of *An. gambiae* and *An. nili*

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Months	<i>An. gambiae</i> HLC Indoor No. of Catches	<i>An. nili</i> HLC indoor No. of catches	Time (hours)
May	1	0	22:00
June	0	3	22:00
July	3	0	23:00
August	0	0	23:00
Sept.	0	0	23:00
Oct.	1	1	3:00

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257 **Table 7:** Outdoor host seeking behaviour of *An. gambiae* and *An. nili*

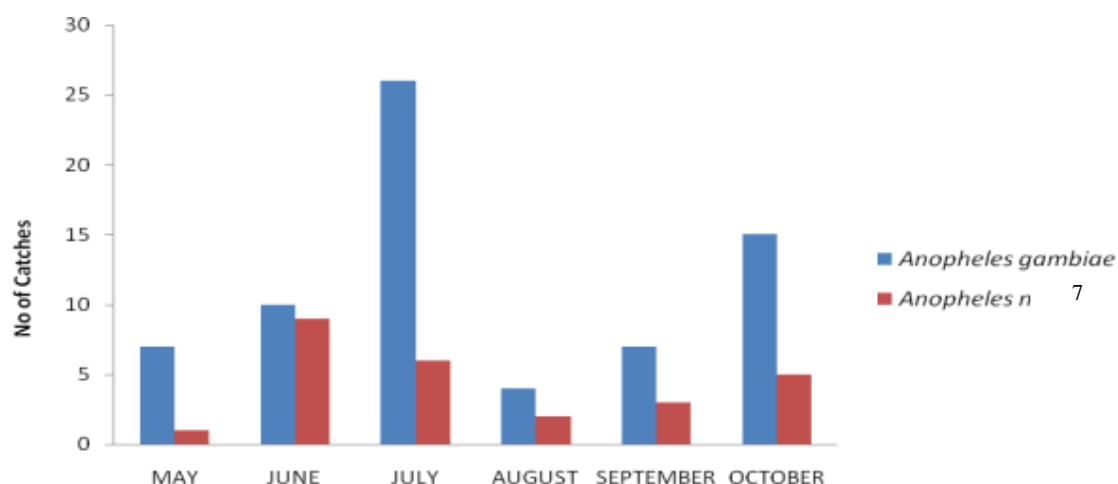
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Months	<i>An. gambiae</i> HLC outdoor No. of Catches	<i>An. nili</i> HLC outdoor No. of catches	Time (hours)
May	4	2	18:00

June	2	3	18:00
July	9	1	19:00
August	1	0	18:00
Sept.	2	1	20:00
Oct.	5	1	18:00

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262 **Figure 2: Variation in monthly collection of anopheles mosquitoes**

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264 **Table 8: Human biting rates of *An. gambiae* sensu stricto and *An. nili* complex**

Months	Species	No. of Nights of Catch	No. of baits		Total catches indoors		Total catches outdoors	Human biting rate		Total
			Indoors	Outdoors	6pm – 10pm	10pm – 6am	6 – 10 pm	Indoors (3+8hrs)	Outdoors (1hr)	
May	<i>An. gambiae</i>	2	1	1	0	0	3	0.5	0.4	0.9
June		2	1	1	1	0	1	0.4	0.1	0.5
July		2	1	1	1	2	9	1.3	1.1	2.4
August		2	1	1	0	0	1	0.5	0.1	0.6
Sept		2	1	1	0	0	2	0	0.3	0.3
Oct		2	1	1	0	1	5	0.5	0.6	1.1
May	<i>An. nili</i>	2	1	1	0	0	2	0	0.3	0.3
June		2	1	1	0	2	3	1	0.4	1.4
July		2	1	1	0	0	1	0	0.1	0.1
August		2	1	1	0	0	0	0	0	0

Sept	2	1	1	0	0	1	0	0	0
Oct	2	1	1	0	0	1	0	0	0

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268 4. DISCUSSION

269 The rainforest zone of Nigeria has been described as a malaria endemic area because of the high level of
 270 human- vector contact, which has been attributed to high density of anopheles mosquito vectors in the
 271 area [1]. Reports from previous studies by [10] showed that *An. gambiae s.l* and *An. funestus* group
 272 constitute the largest proportion of malaria vectors in Nigeria. In this study, *An. gambiae* and *An. nili* were
 273 the malaria vectors found in the study area. *An. nili* was an important malaria vector in the study area.
 274 These findings do not agree with the previous findings by [3,10] which reported that *An. nili* is a
 275 secondary vector. Member of *An. gambiae* complex identify in this study was *An. gambiae s.s*.

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277 Vector abundance increased with increasing rainfall, resulting in the proliferation of anopheles mosquito
 278 populations. The 12.8% of anopheles mosquitoes collected in the study area showed that anopheles
 279 mosquitoes were not predominant in the areas. This could be because anopheles mosquitoes are not
 280 predominant in the urban areas [11]. Collection of samples were most abundant in the month of June
 281 which coincided with the peak of rainy season, however collection of *An. gambiae* was most abundant in
 282 July after two months onset of rain and collection of *An. nili* was abundant in the month of June.

283

284 Large number of anopheles mosquitoes collected in Urua Udofia Street could be due to the location of the
 285 area and its proximity to slow moving streams. This finding agrees with previous studies by Bockererie
 286 [4,10] which reported that anopheles mosquitoes were predominant in villages with proximity to slow
 287 moving streams. *An. nili* was found to be one of the main malaria vectors in the study area.

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289 This study indicates that *An. gambiae s.s* seeks host in outdoor venues in Ewet Offot, Uyo. The greater
 290 proportion of *An. gambiae s.s* in this area leave houses in the morning and rest outdoors. This species
 291 was predominant and the most aggressive vector in the area. The abundance of *An. gambiae* had been
 292 reported in the Southwest Nigeria by [3]. This has been found to be connected with certain factors most
 293 especially the annual precipitation which appears to influence the range and relative abundance of this
 294 species. *Anopheles nili* complex were highly exophagic/exophilic in the study areas. This finding also
 295 agrees with previous findings by [11] which reported that *An. nili* complex are highly exophagic and
 296 exophilic and can bite man as well as other vertebrates in remote areas. However, in this study more than
 297 70% host seeking mosquitoes were collected outdoors in the early evenings.

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299 Biting activities of both *An. gambiae s.s* and *An. nili* commenced effectively at the early part of the night
 300 before the inhabitants retire to bed. *An. gambiae s.s* are considered to exhibit highly endophagic and
 301 endophilic behaviour and to feed primarily on human hosts [2] even though endophagy has been shown
 302 to be a variable trait [3]. In this study, *An. gambiae s.s* was responsible for both indoor and outdoor biting
 303 activities and was found to be highly endophilous compared to *An. nili*. This indicates that this species
 304 preferred to rest indoor after feeding. It was shown that the biting activity of *An. gambiae* complex species
 305 commenced at 1800 hours (outdoor) with the peak biting activity at 2300 hours (indoor) in the July.
 306 However studies conducted by Dandalo [5] reported that the biting activity of *An. gambiae* complex
 307 species commenced at 1900 hours and ceased at 0500 hours with peak biting activity in March [3]
 308 reported that the biting activity of *An. gambiae* complex gradually increased throughout the night with a
 309 peak biting attained three hours before dawn in Kenya.

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311 The biting activity of *An. gambiae* complex is dynamic and varies in response to changes in
 312 environmental factors such as wind and rainfall [13]. On the other hand *An. nili* was more exophagic with
 313 60% of biting activity occurring outdoor. Biting activity of *An. nili* commenced at 1800 hours and peak at
 314 2200 hours and decreased thereafter at 0300 hours in June. In Cameroon the peak biting activity of *An.*
 315 *nili* occurred between midnight and 0100 hours [15]. Takken [15] reported that the biting activity of *An.nili*
 316 can be disrupted by changes in environmental factors during the night, example rain and wind. The peak
 317 biting activity of anopheles mosquitoes in the early hours of the evening 1800 hours shows that the
 318 possibility of outdoor transmission may be high because at this period the inhabitants of the areas are still
 319 active outdoors.

The endophilic behaviour of *An.gambiae* would be a prerequisite for significant contact between the vector species and man. The resting behaviour of *An. nili* species in Ewet Offot, Uyo is mainly exophilic /endophagic. The highest number of *An. gambiae* was collected in July whereas the highest number of *An. nili* collected was recorded in June. Based on the exophilic behaviour of *An. nili* complex at Ewet Offot, Uyo, Residual Insecticidal Treatment of houses would not be ideal for vector control since it is mostly designed to kill indoor resting fraction of malaria vector population. The Human Biting Rates (HBR) recorded for *An. gambiae* was higher than *An. nili* (76.7%). This agrees with previous findings by Awolola [1] which reported similar HBR of *An. gambiae*. The Human Biting Rates (HBR) can be an important factor in the epidemiology of the disease and in estimating the vector-human contact.

5. CONCLUSION

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The results from this study show that *An. gambiae* and *An. nili* co-exist in Ewet Offot, Uyo, Akwa Ibom State. *An. gambiae* is the predominant species in this area. The biting patterns of *An. gambiae* and *An. nili* vary in response to changes in environmental factors such as wind and rainfall. The differences in the Human Biting Rates (HBR) for *An. gambiae* s.s and *An. nili* complex during the six months of sampling exhibit variations in biting activity of these species at Ewet Offot. The peak of biting activity of *An. gambiae* s.s occurred before mid - night at 2300 hours indoor and 1900 hours outdoor in July whereas the peak of biting activity for *An. nili* occurred at 2200 hours indoor and 1800 hours outdoor in June. The resting behaviour of *An. gambiae* s.s at Ewet Offot is endophilic. Low rate of endophagy (0.2) of *An. nili* indicates that a greater proportion of this species in the area is exophilic. The findings from this study contributes to the basic understanding of the distribution of Anopheles mosquitoes with respect to species composition and heterogeneities that could serve as a base- line data for further entomological studies in the area.

Ethical approval was not needed since it was a vector study.

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