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Molecular characterization, seasonality, gonotrophic stages and parity status of malaria vectors in a rural community

Obi, Onyinye Juliet[1], Onyali, Ikechukwu Oliver[1], Ikpeze, Obiora Osegboka[1], Ebuka, Kingsley Ezihe[2*], Nwangwu, Udoka Chukwubuofu[2], Tolulope Oyeniyi[3]

1.Department of Parasitology and Entomology, Nnamdi Azikiwe University Awka, Nigeria
2.National Arbovirus and Vectors Research Centre, Enugu, Nigeria
3.Nigeria Institute of Malaria Research, Yaba, Lagos
* eziheebuka@yahoo.com

ABSTRACT: Malaria which is endemic in Nigeria is caused by *Plasmodium* species that are transmitted through the bite of an infected female *Anopheles* mosquito. A longitudinal study (January-December, 2017) was on molecular characterization, seasonality, gonotrophic stages, and parity status of malaria vectors in rural Oraifite, south-eastern Nigeria. Indoor anthropophagous mosquitoes were sampled by pyrethrum knockdown collection method, and identified morphologically based on appearance of head, thorax, wing colours, and tarsal segments of hind legs of the mosquitoes. The PCR amplification of DNA from legs and wings was used to identify sibling species of mosquito species. Indoor resting density (IRD) was determined as 'number of female mosquitoes collected per room per night' while man-biting rate (MBR) was taken as 'total number of engorged females divided by number of room-occupants the night before collection'. Gonotrophic stages were categorized according to abdominal conditions of mosquitoes as engorged, not-engorged, gravid, and half-gravid while parity was based on presence or absence of ovary-trachea in dissected mosquitoes. Data were subjected to descriptive statistics. Standard error bars on excel bar charts indicated significant differences ($p<0.05$) among variables studied. Both IRD and MBR were computed from derived formulae. Out of 541 *Anopheles* mosquitoes collected, *An. gambiae* comprised 294 (54.35%), *An. funestus* 228 (42.14%), and *An. moucheti* 19 (3.51%), ($p<0.05$). Dry season contributed 11.5% of all collections while rainy season accounted for 88.5% with a peak in September (24.6%). Of 160 *An. gambiae* complex ran on PCR, 125 (78.1%) were amplified as *An. gambiae* sensu stricto. Both IRD (mosquitoes/room/night) and MBR (bites/man/night) for *An. gambiae* s.l, *An. funestus,* and *An. moucheti* were (0.42; 0.2), (0.3; 0.14), and (0.02; 0.01) respectively. Of all 541 *Anopheles* species, 429 (79.3%) were engorged were, 41 (7.6%) not- engorged, 39 (7.2%) half-gravid, and 32 (5.9%) gravid. Monthly variation in parity rates of *Anopheles gambiae* complex was significant ($p<0.05$) but all *An. gambiae*, *An. funestus*, and *An. moucheti* collected in August were parous, with respective parity rates of 83, 69, and 57%. High percentages of engorged and parous *Anopheles* species in this study indicated intense activity of the malaria vector *An. gambiae* s. s in Oraifite.

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Keywords: *Anopheles gambiae*, *An. funestus, An. moucheti*, PCR, *An. gambiae* s.s, Oraifite

INTRODUCTION

Malaria is a preventable but life-threatening disease caused by *Plasmodium* species that are transmitted to people through the bites of infected female *Anopheles* mosquitoes. Generally, transmission of mosquito-borne diseases to humans is achieved through blood-feeding activities of mosquito vectors (Scott and Takken, 2012). Malaria vector efficiency is due mainly to the anthropophilic biting behaviour of the female *Anopheles* species (White *et al.,* 2011). Globally, there were an estimated 229 million malaria cases in 2019 in 87 malaria endemic countries which shows progress from 238 million in 2000 (WHO, 2020). In tropical Africa, *Anopheles gambiae* sensu lato (s.l) and *An. funestus* sensu stricto (s.s) are the most efficient human malaria vectors while *Plasmodium falciparum* is the most virulent malaria parasite (Sinka *et al*., 2010). *Anopheles gambiae* sensu lato (s.l) has been reported as the main malaria vector (Onyido *et al.*, 2011; Onyido *et al.*, 2014) and the predominant species of the indoor biting mosquitoes (Onyido *et al.*, 2016) in Anambra State of Nigeria.

The mosquito's gonotrophic cycle which starts with blood-feeding and ends with egg-laying is

temperature-dependent and continues throughout the mosquito's life span (Saifur, 2012). During blood meals, the female mosquito can ingest pathogens which could disseminate into the body, passing first into the mid gut and crossing intestinal barrier to amplify in the haemocoele before reaching the ovaries and eventually salivary glands. Therefore as the percentage of parous females increases with the age of mosquito population, there would be potential increase in transmission risks. Since the year 2000, use of insecticide-treated nets (ITNs) and long-lasting insecticidal nets (LLINs), as well as indoor residual spraying (IRS), and artemisinin-based combination therapy have significantly reduced prevalence of *P. falciparum* infection and incidence of clinical malaria in tropical Africa (Bhatt *et al*., 2015).

Molecular characterization, and thus the detection of variation as a result of differences in either DNA sequences or specific genes or modifying factors of the malaria vector species is critical to determining the host-feeding and resting behaviours which enable vectors by-pass the most common malaria control interventions like ITNs, LLINs and IRS which are targeted around indoor and night-time biting anthropophilic *Anopheles* species (Killen, 2014). Despite impressive progress made towards the control and elimination of malaria using LLINs and IRS, malaria remains the leading cause of morbidity and mortality in the tropics, with an estimated mortality of 435,000 individuals in 2017 (WHO, 2018). Data from molecular characterization will improve or even allow for elucidation of phylogeny, and provide the basic knowledge for understanding taxonomy, domestication and evolution of species (Nwakanma *et al*., 2003). Information from molecular markers or DNA sequences will also provide the basis for better malaria vector control approaches. The present study was therefore focused on molecular characterization, seasonality, and gonotrophic and parity statuses of malaria vectors at Oraifite, a rural malaria-endemic and bustling community in Anambra State Nigeria. Findings from this study will help in evidence-based policy decision that would strengthen the control of malaria and malaria vectors in the study area, and elsewhere in Anambra State and Nigeria in general.

MATERIALS AND METHODS

Study area:

The study was conducted in Oraifite (Latitude 5.56^0N - 6.03^0N and Longitude 6.9^0E-6.86^0E) Ekwusigo Local Government Area of Anambra State. Oraifite, with a population of 42,346 (NPC/FRN, 2006) is in the rain forest belt of Nigeria and enjoys equatorial tropical climate that is characterised by rainy season (April-October) and dry season (November-March). This rural environment is characterised by suitable mosquito larval habitats that ensure successful breeding and maintenance of different mosquito species throughout the year.

Study design:

The longitudinal study was carried out during the dry and wet seasons of the year, every first five days in the months between January 2017 and July 2018. Indoor resting and feeding mosquitoes were sampled using pyrethrum knockdown collection (PKC) method.

Ethical considerations:

Ethical approval (Ref: COOUTH/AA/VOL1.025) was obtained from The Ethics and Research Committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH) Awka, Anambra State. Advocacy to Oraifite leaders of thought, followed-up with community mobilization and sensitization helped the researchers to obtain informed consent of household heads and residents that participated in the field study.

Mosquito sampling:

Indoor resting and biting adult mosquitoes were sampled from twenty-four houses purposefully selected (three houses from each of the eight communities) in Oraifite. Mosquito sampling was done five days per month from January to December of 2017 by PSC method between 6:00 and 9:00 hours local time (WHO, 1995). The knocked down mosquitoes were routinely preserved in the Department of Parasitology and Entomology laboratory, Nnamdi Azikiwe University, Awka.

Mosquito identification:

Morphological identification to species level was according to Coetzee (2020) based on appearance of the head, thorax, wing colours, and tarsal segments of hind legs. Molecular identification was done at the Nigerian Institute of Medical Research Lagos, according to Scott *et al.* (1993) when PCR amplification of DNA from legs and wings of 160 mosquitoes were used for identification of sibling species.

Determination of indoor resting density (IRD) and man-biting rate (MBR) of mosquitoes:

The IRD of mosquitoes was taken as the number of female mosquitoes per number of room per number of nights while MBR, expressed as the number of bites an occupant of room receives from a vector per night, was computed indirectly as the total number of engorged mosquitoes collected each day divided by

total number of occupants of the room on the night before collection (Ezihe *et al*. (2017).

$$IRD = \frac{(Total\ number\ of\ mosquitoes\ collected\ indoors) \div (Total\ number\ of\ rooms)}{Total\ number\ of\ nights}$$

$$MBR = \frac{(Total\ number\ of\ engorged\ mosquitoes) \div (Total\ number\ of\ room\ occupants)}{Total\ number\ of\ nights}$$

Determination of abdominal stages of mosquitoes:

All collected female mosquitoes were counted and categorized on the basis of abdominal stage as engorged (blood-fed), not engorged (unfed), gravid, and half-gravid (WHO, 1975). The females were kept individually in coded vials containing silica gel at laboratory temperature for subsequent examinations. Each vial was labeled to show mosquito species, date of collection, and identification mark of the house.

Determination of parity rates of mosquitoes:

Abdomens of about 40% of female mosquitoes were dissected to determine their parity rate based on presence or absence of ovary-trachea filaments (Detinova, 1962). Ovaries were dissected in a drop of phosphate buffered saline (PBS) solution on a slide, and examined under an optical microscope (Olympus) at ×40 magnification. Parous and nulliparous statuses were based on the presence or absence of ovary-trachea filaments.

Data Analysis:

Data were collated and subjected to descriptive statistics. Relative frequency of each species was calculated against the total catch. Further analysis was done with Bar Charts in MS Excel. Standard error bars on bar charts indicated significant differences ($p<0.05$) among variables studied. The IRD and MBR were computed from respective derived formulae.

RESULTS AND DISCUSSIONS

Out of a total of 541 *Anopheles* mosquitoes collected indoors (Table 1), *An. gambiae* comprised 54.35%, followed by *An. funestus* (42.14%) and *An. moucheti* with 3.51% ($p<0.05$).

Table 1: Percentage compositions of mosquito species collected

Mosquito species collected	No.	%
Anopheles gambiae	294	54.35
Anopheles funestus	228	42.14
Anopheles moucheti	19	3.51
Total	541	100.00

Seasonality in the study area is characterized with a period of rainfall (April-October) and a period of dryness (November-March). The dry season contributed about 11.5% of all mosquitoes collected while rainy season accounted for about 88.5%, with a peak of 24.6% in September (Figure 1). Rainfall has been reported is a key factor in abundance of malaria vectors, which in turn enhances malaria transmission in several countries (Thomson *et al*., 2005). From this study, there was a fluctuation in *Anopheles* mosquito abundance from wet to dry season which was also evident in the findings of Ezihe *et al.* (2017).

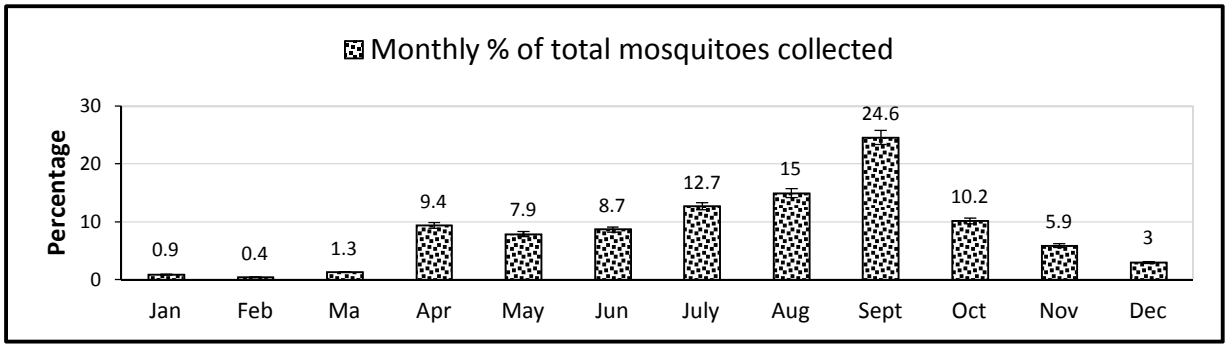

Figure 1: Monthly distributions (%) of mosquitoes collected indoors

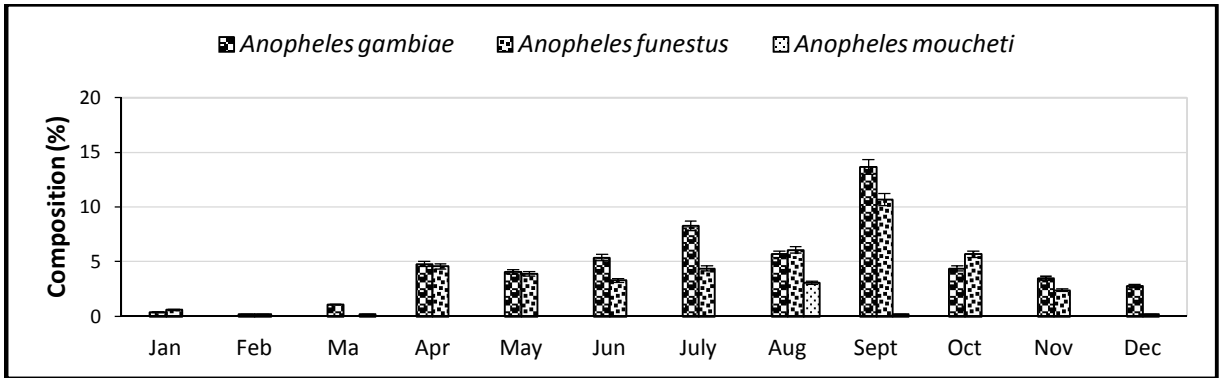

Figure 2: Monthly composition (%) of *Anopheles* species collected indoors

Monthly contributions of mosquito species showed that all the three species were collected in August (Figure 2). It was also observed that *Anopheles gambiae* and *An. funestus* were collected throughout the year (except in March when *An. funestus* was not recorded) but *An. moucheti* was collected mostly in August.

One hundred and twenty-five (125) or 78.1% out of the 160 *An. gambiae* complex subjected to PCR were amplified, and thus identified as *An. gambiae* sensu stricto (M&S) forms (i.e., *An. gambiae* s.s and *An. coluzzii* in the ratio of 80:20) leaving out 35 (21.9%) neither amplified nor identified (Figure 3).

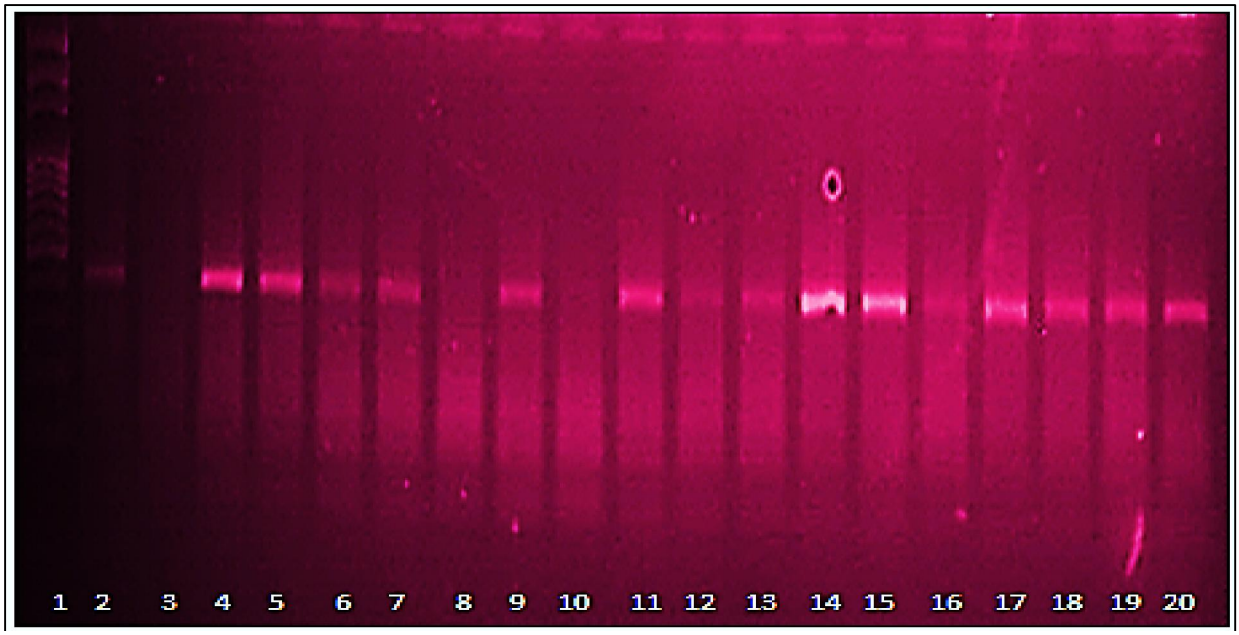

Figure 3: The PCR product of Agarose gel electrophoresis for *Anopheles gambiae* complex. Lane 1 is DNA ladder.

Lanes 2 and 3 were positive and negative controls respectively. Lanes 4,5,6,7,9,11,12,13,14,15,16,17,18,19 and 20 were products of *An. gambiae* s. s (M&S forms) while Lanes 8 and 10 were unamplified and thus unidentified.

This molecular identification of sibling species in Oraifite is similar to the findings of Chukwuekezie *et al*. (2020) where the same two sibling species *An. gambiae s.s* and *An. coluzzii* were reported. In the present study, *An. gambiae* s.s was significantly

more abundant than *An. coluzzii* though the percentage was not determined. Reports from other parts of southern Nigeria (Awolola *et al.*, 2005; Onyabe *et al.,* 2003) showed that *An. gambiae* s.s is a predominant and widely distributed species when compared with *A. coluzzii* but Chukwuekezie *et al.* (2020) reported otherwise from Ebonyi State. Nevertheless, information on molecular characterization of the *Anopheles gambiae* s.l DNA offered a good basis for better control approaches and can quickly help to check whether changes in alleles or allele frequencies have taken or are taking place.

Indoor resting density and man-biting rate of *Anopheles gambiae* complex (Table 2) revealed that *An. gambiae* s.l. had the highest IRD of 0.42 mosquitoes/room/night and MBR of 0.2 bites/man/night. These observations were lower than the findings in Enugu State, where *An. gambiae* had an IRD 0.66 mosquitoes/room/night and a MBR 3.9 bites/man/night were reported (Ezihe *et al.*, 2017). The result was also far below the findings in Bayelsa State (Ebenezer *et al.*, 2013) where *An. gambiae* had MBR 8.7 bites/man/night and IRD 20.5 mosquitoes/room/night. This suggest that *An. gambiae* mosquitoes are biting less in the study area which may justify that a form of intervention may have been put in place but not effectively carried out in the study area.

The overall prevalence of gonotrophic stages of collected mosquitoes (Figure 4) revealed that engorged mosquitoes were highest (79.3%) while gravid ones were least (5.9%). There was similarity in the trends of gonotrophic stages among the three species. There was no significant difference in gonotrophic stages between *An. gambiae* s.l and *An. funestus* s.l but those of *An. moucheti* differed significantly.

Table 2: Indoor-resting density and man-biting rate of adult mosquitoes collected in twenty-four (24) nights from twenty-four (24) rooms with fifty-one (51) occupants

Months	All mosquitoes collected (no.)	Engorged mosquitoes collected (no.)			
		Total	*An. gambiae*	*An. funestus*	*An. moucheti*
January	5	3	2	1	0
February	2	1	1	0	0
March	7	4	4	0	0
April	51	46	22	24	0
May	43	36	19	17	0
June	47	35	22	13	0
July	69	51	32	19	0
August	81	60	27	22	11
September	133	105	60	45	0
October	55	49	22	27	0
November	32	24	16	8	0
December	16	15	15	0	0
Total no. (%)	541(100.0)	429 (79.29)	242 (44.73)	176 (32.53)	11 (2.03)
Engorged (%)			56.41	41.03	2.56
Indoor resting density (IRD)		0.74	0.42	0.30	0.02
Man biting rate (MBR)		0.35	≈ 0.2	≈ 0.14	≈ 0.01

$IRD\ of\ all\ engorged\ mosquitoes = \frac{(429) \div (24)}{24} = \frac{17.87}{24} = 0.74$

$IRD\ of\ An.gambiae = \frac{(242) \div (24)}{24} = \frac{10.08}{24} = 0.42$

$IRD\ of\ An.funestus = \frac{(176) \div (24)}{24} = \frac{7.33}{24} = 0.30$

$IRD\ of\ An.moucheti = \frac{(11) \div (24)}{24} = \frac{0.458}{24} = 0.019 \approx 0.02$

$\text{MBR}\ of\ all\ mosquitoes = \frac{(429) \div (51)}{24} = \frac{8.41}{24} = 0.350$

$MBR\ of An\ gambiae = \frac{(242) \div (51)}{24} = \frac{4.75}{24} = 0.197 \approx 0.2$

$\text{MBR}\ of An.funestus = \frac{(176) \div (51)}{24} = \frac{3.45}{24} = 0.144 \approx 0.14$

$\text{MBR}\ of An\ moucheti = \frac{(11) \div (51)}{24} = \frac{0.216}{24} = 0.009 \approx 0.01$

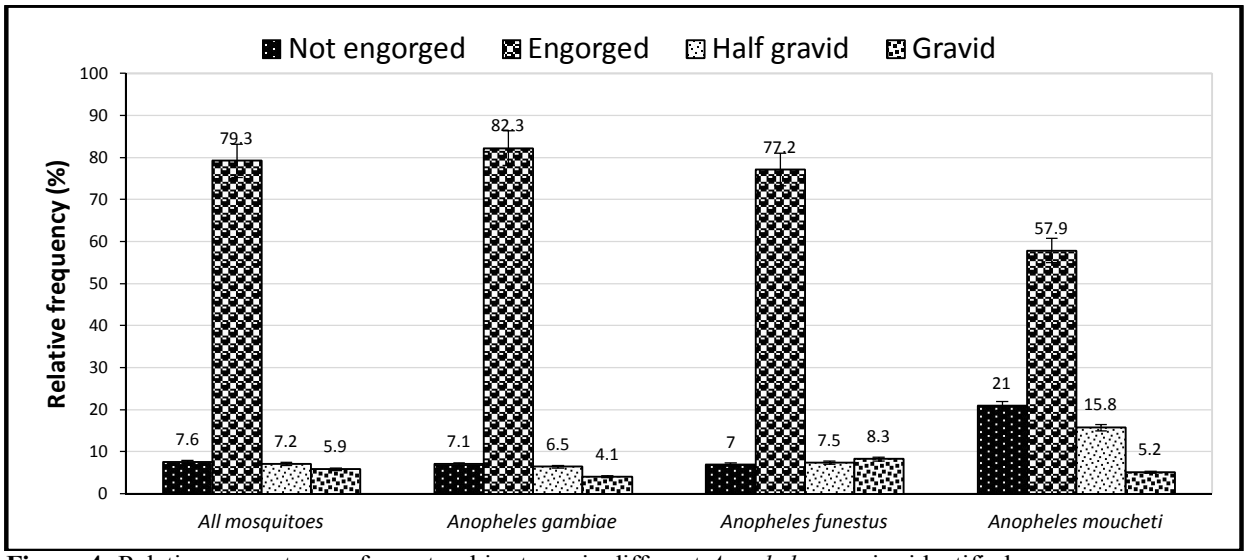

Figure 4: Relative percentages of gonotrophic stages in different *Anopheles* species identified

The high percentage of engorgement witnessed in the present study may be an indication that a greater percentage of mosquitoes may have blood meal and as such, there was a high tendency of infected mosquitoes transmitting *Plasmodium* species, hence malaria infection. It was similarly observed (Ezihe *et al.*, 2017) that about 74.4% of *An. gambiae* mosquitoes collected indoors in their study in Enugu State were engorged. Similar observations were made in Abeokuta (Adeleke *et al.,* 2010) where almost 84% of *An. gambiae* collected indoors were either engorged or gravid.

With respect to monthly gonotrophic stages of the *Anopheles* species (Figure 5), there was similarity in trends of prevalence of gonotrophic stages *An. gambiae* and *An. funestus*, which differed significantly from that of *An. moucheti*. There was insignificant difference between gonotrophic stages of *An. gambiae* and *An. funestus* as far as the gravid, half-gravid and not-engorged female mosquitoes were concerned. The highest percentages of engorged *An. gambiae* and *An. funestus* were collected in the month of September during the peak of rains. This is in conformity with the finding (Ezihe *et al.,* 2017) that people tended to be indoors when it rained (especially at peak periods) thereby aiding indoor mosquito bites and potential malaria transmission.

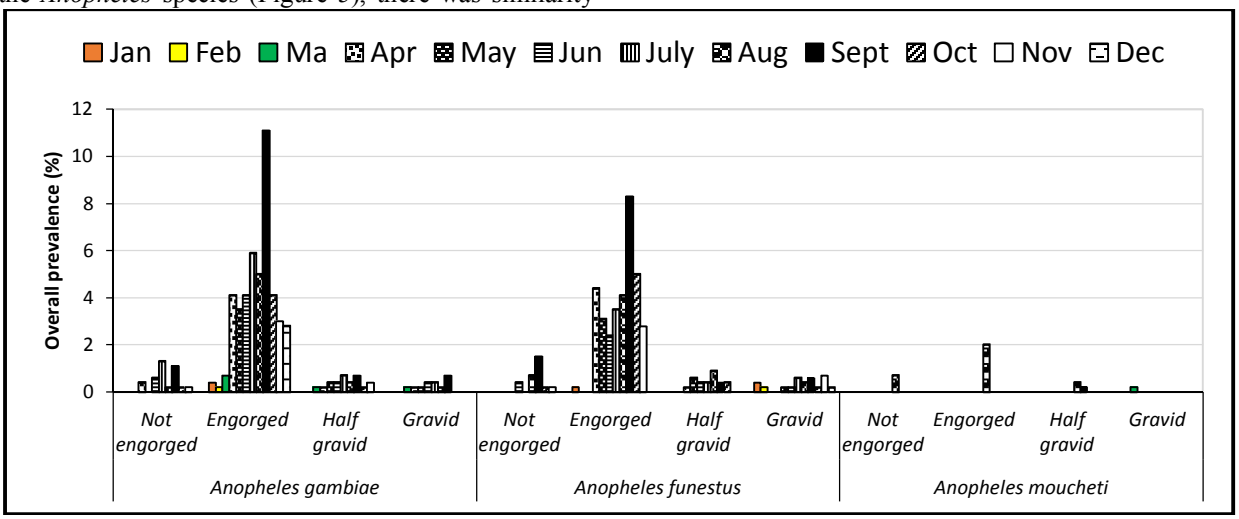

Figure 5: Monthly gonotrophic status of *Anopheles* species

The *Anopheles* species collected in the month of August (Figure 6) were all parous. The variability in parity rates between all the months was statistically significant (p<0.05). *Anopheles gambiae* collected during the study period from 24 households showed a higher parity rate of 83% followed by *An. funestus* (74%) and *An. moucheti* (57%). All the vectors collected in this study were parous for the month of August as this in tandem with the findings of Taye *et al.* (2017) that the highest abundance of parity in his study was in the month of August.

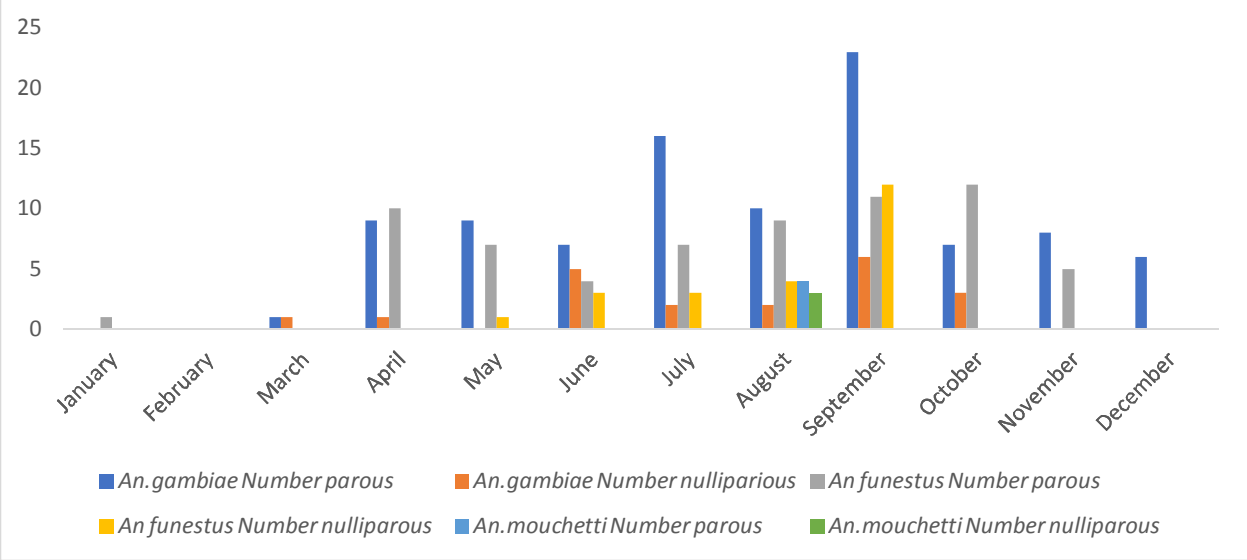

Fig 6: Parity rate of the *Anopheles* species collected indoors

Consequently, there was high rate (75%) of parous females in the study area which suggest that majority of the mosquitoes were able to obtain a blood meal and complete at least one or more gonotrophic cycles and thus indicates high survival rate and high vectorial capacity for disease transmission, as only parous flies could transmit diseases. Also, the majority of the mosquitoes being parous indicate that there were older populations. The majority of parous mosquitoes collected points towards the absence of intervention or reduced application of vector control measures and interventions in the community. This finding is also in accordance with the recorded of parous mosquitoes collected indoors by Uttah *et al.* (2013) and contrasted with Adeleke *et al.* (2010) who recorded higher percentage of nulliparous mosquitoes which he explained could be as a result of high productivity of their breeding sites.

Conclusion

The availability of *An. gambiae, An. funestus* mosquitoes in the study area which are the primary transmitters of malaria parasite has shown that the inhabitants were exposed to the bites and nuisance of these mosquitoes and possibly disease transmission. Also, malaria transmission in Oraifite of Anambra State could be mainly by *An. gambiae s.s* or *An. coluzzii* which were seen to be excellent transmitters of *plasmodium* parasite. Other species may be playing minor role. If the malaria vectors are not controlled, the effect will be disastrous. One thing is certain that when a child or any community members get sick, loss of productive time in school or work will increase, money will be spent on treatment, caregiving will resume with its associated costs and government expenditure will increase. More health education on the vector ecology especially breeding habitats needs to be entrenched amongst the youths in the community so they can be involved in community sanitation and sand filling the breeding habitats.

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Corresponding Author:
Ezihe, Kingsley Ebuka
E-mail: eziheebuka@yahoo.com

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