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Entomological indices of *Anopheles gambiae* sensu lato at a rural community in south-west Nigeria

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Abstract

Background & objectives: Investigations were conducted to obtain key entomological indices of *Anopheles gambiae* s.l. at Igbo-Ora, a rural community in south-west Nigeria.

Methods: Mosquitoes were caught daily for a week from rooms where tenants had slept the previous night in each of the four months June, July (2001), and August, September (2002). *Anopheles gambiae* s.l. sibling species were PCR-identified, the blood meal origin was determined by direct ELISA, and the circumsporozoite antigen by sandwich ELISA. Mean weekly rates were calculated.

Results: The mean human biting rates were 0.90 and 1.6 in 2001 and 2002 respectively. The mean weekly anthropophilic rates for *An. gambiae* s.l. were 82 and 86% in 2001 and 2002 respectively; they were high in *An. gambiae* s.s., *An. arabiensis* and non-identified species in the complex. The mean weekly circumsporozoite rates were 6.70% in 2001 and 6.30% in 2002. The mean weekly entomological inoculation rates (EIR) were 4.95 and 5.05 in 2001 and 2002 respectively; the seasonal (6-month) rates were high: 128.7 in 2001 and 131.3 in 2002, compared to data from other rural communities on the continent.

Interpretation & conclusion: The implications of these findings on the role of *An. gambiae* s.l. in the holoendemicity of malaria at Igbo-Ora are discussed.

Key words *Anopheles gambiae* s.l. – entomological indices – Igbo-Ora – malaria – Nigeria

Introduction

The factors that influence the role of mosquitoes in malaria transmission include host preference, feeding and resting behaviour, adult longevity and density as they determine the degree of anthropophily, the human biting rate and host location strategy[1]. The dominance and exceptional stability of *Plasmodium falciparum* life-cycle with mean inoculation rates consisting of hundreds of infective bites per man per year, consistently observed over large areas characterize malaria epidemiology in sub-Saharan Africa[2]. Significant variations in vector biology, within and between countries have been reported in malaria epidemiological studies[3]. It is now generally agreed that a clear understanding of the detailed epidemiology of the disease is a pre-requisite to effective malaria control in the African sub-region[4]. To appraise the disease burden at Igbo-Ora, a rural community in the forest-savannah woodland ecocline, south-west Nigeria, an integrated study on malaria immunology and parasitology was initiated in 1991; the entomology and socioeconomic components commenced in 2001. The present report is from the first phase of long-term studies that began in 2001 on the role of *Anopheles gambiae* s.l. (Diptera : Culicidae) in the epidemiology of malaria at Igbo-Ora and the present study focuses on the determination

of human biting, anthropophilic and entomologic inoculation rates.

Material & Methods

Study area: Igbo-Ora is a rural town, located 70 km west of Ibadan in the forest-savannah woodland ecocline south-west Nigeria (Fig. 1). It covers an area of approximately 100 km^2, with a population of about 60,000 mainly of the Yoruba ethnic group[5]. Contrary to the description by Lawrence[6], the area is strewn with streams that serve as larval breeding pools for *An. gambiae* s.l. The surroundings of habitations remain permanently bushy with thick and tall grasses. Water pots were located at the rear of the houses and in sleeping rooms, although these are usually not the preferred breeding sites of *An. gambiae* s.l.[7], they may be used, particularly in the dry season, when preferred sites are few[8]. Mosquitoes move easily into houses through the corridors, which are permanently open at both ends of the building. Goats, sheeps, dogs and chickens utilise the houses as cattlesheds. Latrines are rare or non-existent and the drainage system is very poor, stagnant pools are common around the houses. Water supply remains a major problem; ponds, hand-dug wells and a few boreholes donated by UNICEF provided what the villagers referred to as 'hard water'. These donors permanently maintained the few functional wells and boreholes in support of the guinea worm eradication programme[9]. Water pots kept at the backyard were efficient breeding pools for human disease vectors, including *An. gambiae* s.l. Malaria incidence prevalent rates of 26–70% in children of the age group, <1–15 years, and 42% in adults were recorded at Igbo-Ora during the study period[10,11]. The major activities were subsistence farming (by 80% of inhabitants), small-scale cocoa and tobacco farming, and petty trading among the middle class citizens. Malaria is endemic at Igbo-Ora with a 6-month transmission season (May–October) reaching its peak in August.

Methods: Mosquitoes were collected at Igbo-Ora, during the transmission seasons in 2001 (June–July) and 2002 (August–September). Collections were undertaken daily for one week in each month from houses voluntarily made available by the landlords/tenants for the study. Four villages (Ajegunle, Igbole, Igbo-Ora and Sagaun) were covered in the exercise. Adult resting mosquitoes, identified as anophelines by their resting position, were caught with a simple aspirator, modified from the mouth aspirator model of Coluzzi and Petrarca[12]. All collections were made

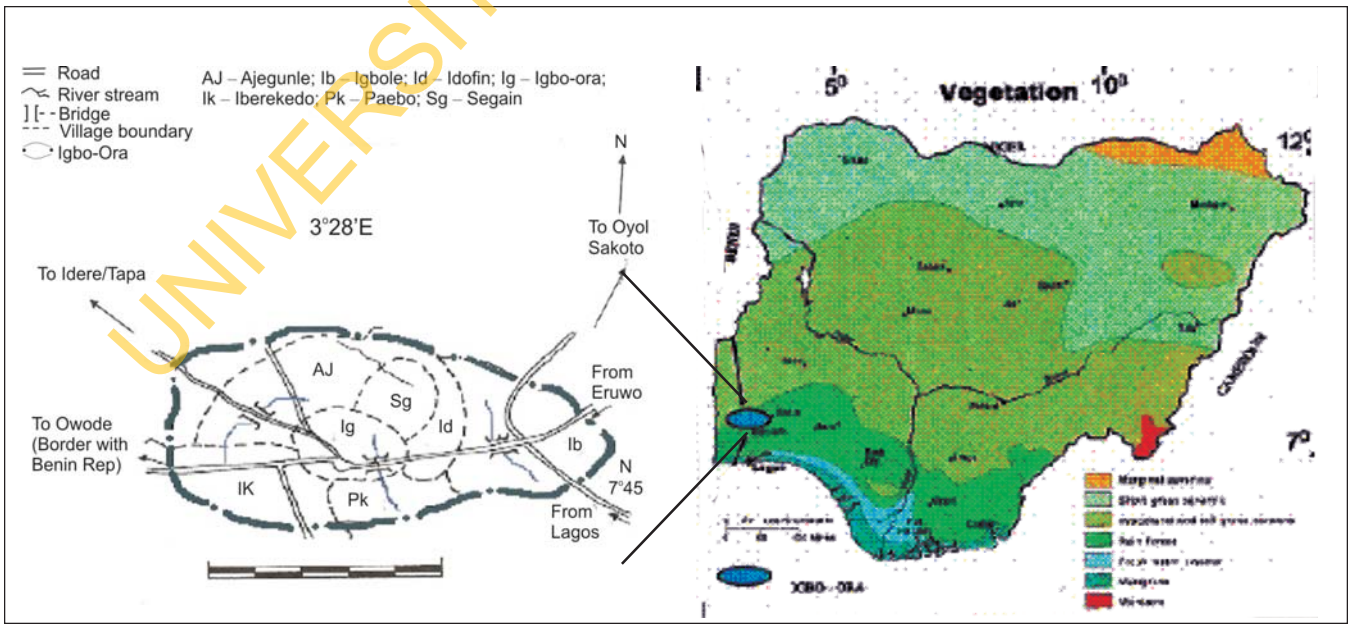

Fig. 1: The mosquito collection sites at Igbo-Ora, Nigeria

in rooms where people had slept the previous night, in the morning (0800–1100 hrs), before they vacated the rooms to ensure that no mosquitoes that had fed outdoors were collected. Furthermore, exophily after feeding had been recorded in *An. gambiae* s.s.[13,14].

Upon capture, the mosquitoes were stored in plastic tubes, capped with nylon netting, one tube per room or house, depending on the mosquito density. For subsequent sampling sessions, these houses and rooms were retained for the rest of the study period. Mosquitoes were put on ice for about 10 min to weaken them and subsequently transferred into a petri dish for proper morphological identification, with a hand lens. They were sorted by the appearance of abdominal blood repletion stage. The significant reduction or complete absence of scales on the abdominal sternites and usually tergites and the smoothly rounded posterior end of the scutellum were used to confirm members of the genus *Anopheles*[15]. Patterns on wings were used to separate *An. gambiae* s.l. from *An. funestus*, *An. moucheti* and *An. nili*[16]. They were classified for gonotrophic state, unfed, fed, half-gravid and gravid, based on abdominal appearance[17]. These were then transferred into a labelled 50 ml corning tube, kept under the shade for 3–6 h and fixed in Carnoy's fixative (100% ethanol: glacial acetic acid 3:1) contained in screw top 1 ml Eppendorf tubes and stored on ice. These were re-sorted by a pair of flexible-tipped forceps in a petri dish under a binocular dissecting microscope (Bunton Instruments Co. Inc., Rockville, MD, USA), and subsequently dissected into various parts (head-thorax, blood meal, abdominal carcass and ovaries) based on their gonoptrophic stages. These were stored at –70 to –20°C in 0.2 ml Eppendorf tubes with Carnoy's fixative for PCR analyses. Analyses were undertaken at the Malaria Research and Training Centre (MRTC), Bamako, Mali. The general information about the tenants and collection venue, date and hospital number were recorded, and later entered into an Excel Database.

DNA source: The carcasses of abdomen and crushes from head-thorax were used for CSP-ELISA and DNA extraction, and amplification for the species identification of the *An. gambiae* complex.

DNA extraction technique: Mosquito parts were crushed in 50 µl of 0.5M fly grinding buffer (0.1M NaCl + 0.2M Sucrose + 0.1M Tris HCl (pH 9.1) + 0.05M EDTA + 0.05% SDS + sterile water) and centrifuged for 2 min with Eppendorf centrifuge 5417 R/5115C/5117C (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Cells were lysed for 30 min at 65°C in a precision water bath (Jouan Inc., Winchester, VA, USA), 7 µl of 8M potassium acetate was added to each tube and incubated for 30 min at 4°C, then centrifuged at 14000 rpm for 15 min. The supernatant was transferred to a newly labelled tube; to precipitate the DNA, 100 µl of 100% ethanol was added to each tube, and then incubated at room temperature for 5 min before centrifuging again for 15 min at 14000 rpm. The DNA was purified with 70% ethanol and centrifuged for 5 min at 14000 rpm. The tubes were air-dried at room temperature and the DNA re-suspended in 50–100 µl of sterile water.

Polymerase chain reaction test: The primers for *An. gambiae* s.l. species identification were purchased from Invitrogen. DNA amplifications were done with PTC-100HB and PTC-200 DNA Engines (MJ Research, Inc. Watertown, MA, USA); the PCR method by Scott *et al*[18] was used.

ELISA tests: These were conducted in disposable unmarked 'U' bottom flexible polyvinyl chloride microtiter plates that were sealed with plate sealers (Dynex Technologies Inc., Chantilly, VA, USA). Mosquito parts were crushed with Kontes pestles (Kontes Glass Company, Vineland, NJ, USA) on a vortex (Scientific Industries Inc., Bohemia, NY, USA) in 1.5 µl flat cap Eppendorf tubes, placed on floating microcentrifuge tube racks (Nalgene Nunc International, USA). The ELISA technique was performed according to methods adapted from Burkott *et al*[19], Beier *et al*[20] and Wirtz *et al*[21]. Antigen-antibody reactions were simulated in immunoplates with mosquito parts, human and *P. falciparum* specific labelled or unlabelled isolates. The plates were de-

veloped and reactions read with the naked eye.

Sandwich ELISA for circumsporozoite antigen detection in mosquitoes: A total of 1077 anophelines' head-thoraxes were used for CSP-antigen detection by the sandwich-ELISA technique as described by Wirtz *et al*[21]. Loss of samples was incurred due to handling, only 1067 samples were tested for the presence of CSP antigen. All head-thoraxes of mosquitoes identified morphologically as *An. gambiae* s.l. were analysed for the presence of CSP antigen, so as to determine the vector population's sporozoite rates. The sporozoite antigen was obtained by crushing one mosquito head-thorax in 50 µl of blocking buffer Np_{40} and 200 µl of blocking buffer [0.5% casein + 0.1N NaOH + PBS (pH 7) + Thimersol ($C_9H_9HgO_2SNa$) + Phenol red]. The microplates were coated with unlabelled monoclonal antibody [phosphate buffer saline (PBS) (*Dulbecco*) + unlabelled Pf2A10-CDC-01 (Kirkegaard & Perry Lab, Maryland, USA)] for 30 min at room temperature. The non-coated spaces were blocked for 1 h with 200 µl of blocking buffer at room temperature; later, 50 µl of sporozoite antigen and controls were bound to each corresponding well for 2 h. The plates were washed twice with washing solution (PBS-Tween 20) and the antigen sandwiched with 50 ml of peroxidase-labelled monoclonal antibody [BB + HRP labelled Pf 2A10-01 (Kirkegaard & Perry Lab, Maryland, USA)] for one hour at room temperature. Excesses were washed off thrice and 100 µl ABTS peroxidase developer (Peroxidase A + Peroxidase B substrates) added to each well. Plates were read after 30–60 min with the naked eye and records were entered on test sheets based on the strength of colouration. These plates were again washed off thrice and 100 µl of phosphatase developer added, then read with the naked eye and recorded after at least 1 h.

Direct ELISA test for blood meal determination: The blood meal remnants of 356 fed and half-gravid mosquitoes identified morphologically as *An. gambiae* s.l. were extracted and tested for their origin (human, bovine, caprine and avian) by direct ELISA, in order to calculate the anthropophilic rates. Antisera were not available for the blood meal analyses of mosquitoes that fed on animals. The blood meal antigen was obtained by crushing one half-gravid or fed mosquito's blood meal remains in 250 µl of PBS. The micro-plates were sensitized with 50 µl of the antigen and incubated at room temperature for three hours. They were washed twice with PBS-Tween 20 before 50 µl of double conjugate (solution D + human peroxidase + bovine phosphatase) were added and incubated for one hour at room temperature. The plates were washed thrice, and 100 µl of peroxidase developer added to each well. Reading and recording were done as described earlier. Three other washes and addition of 100 µl of phosphatase developer preceded reading and recording.

The weekly human biting rate was an estimate of the ratio of fed and half-gravid anophelines, showing human blood in their abdomen, caught in a room and the number of humans that slept in the same room for a period of one week. Anthropophilic rates were estimated as percent of fed mosquitoes that had taken a human blood meal (that had human serum in their midgut at the time of capture) to the total number of fed and half-gravid mosquitoes tested. Sporozoite rates were percent of *Anopheles* mosquitoes with circumsporozoite protein(CSP) antigen in their salivary glands/head-thorax region to the total number of mosquitoes under investigation. The entomologic inoculation rate (EIR) normally expresses the intensity of malaria transmission, which is the product of the vector biting rate and the proportion of mosquitoes infected with sporozoite-stage malaria parasites. The 6-month seasonal inoculation rate was obtained by multiplying the mean weekly rate by 26.

Results

A total of 164 houses were visited in four villages and at least one *An. gambiae* s.l. was caught in each of 91 houses during the sampling period, the percentage of houses with mosquitoes was in the range, 50–60%, in each of the two years. The mean vector population density was 11 anophelines per house with a mean

human population density of approximately two persons per house and estimated mean weekly human biting rates of 0.90 and 1.6 in 2001 and 2002 respectively (Table 1). Gonotrophic stage evaluation of the 989 morphologically-identified *An. gambiae* s.l. showed that 61.82% were gravid (Table 2). From the 364 fed and half-gravid females tested for blood meal source, 304 (83.52%) fed on humans and 50 (13.74%) on undetermined source; 10 (2.74%) blood remnants were lost during storage and crushing. Mean weekly anthropophilic rates were 82 and 86% in 2001 and 2002 respectively (Table 3). Although, *An. arabiensis* constituted only 7.58% of the *An. gambiae* complex, its anthropophilic rate was 88%, while that of *An. gambiae* s.s. was 87% and the non-identified species in the *An. gambiae* complex, 81% (Table 4).

From 1067 specimens tested for the presence of CSP antigen, 1002 (94%) were negative for *P. falciparum*

Table 1. Weekly human biting rates of *An. gambiae* s.l. at Igbo-Ora

Variables	Sampling period					
	2001			2002		
	June	July	Total	August	September	Total
Number of houses visited	33	52	85	48	31	79
Number of harbouring *Anopheles*	13	32	45	31	15	46
% Houses with *Anopheles*	39.4	61.5	52.9*	64.6	48.4	58.2*
Number of *Anopheles* caught	136	354	490	323	176	499
Mean number per house	10.5	11	10.9*	10.5	11.7	10.9*
Number of persons in houses	59	139	198	75	40	115
Number fed + half-gravid *Anopheles*	52	128	180	124	60	184
Weekly human biting rates	0.88	0.92	0.90*	1.65	1.5	1.6*

*Mean values.

Table 2. Feeding and reproductive status of *An. gambiae* s.l. at Igbo-Ora

Variables	Sampling period					
	2001			2002		
	June	July	Total	August	September	Total
Total No. of females anophelines	136	354	490	323	176	499
Unfed *Anopheles*	6	34	40	13	26	39
Fed *Anopheles***	39	96	135	93	45	138
Half-gravid *Anopheles*†	13	32	45	31	15	46
Number fed + half-gravid *Anopheles*	52	128	180	124	60	184
Percent of fed *Anopheles*	38.2	36.2	36.7*	38.4	34.1	36.2*
Gravid *Anopheles*‡	78	192	270	186	90	276

*Mean values;**Fed *Anopheles* had their abdomen filled with blood meal and appeared reddish; †Half-gravid *Anopheles* had their abdomen half full of blood meal/eggs, and appeared reddish/whitish; ‡Gravid *Anopheles* had their abdomen full of eggs and appeared whitish.

sporozoites, while 65 (6%) were positive, as indicated by the presence of the CSP antigen in their head-thorax (Table 3). The CSP antigen distribution among the sibling species correlated with sibling species abundance; *An. gambiae* s.s. yielded 75.4% CSP positives compared to 7.7% in *An. arabiensis*. Mean sporozoite rates in 2001 and 2002 were in the range, 6.3–6.7%. Mean weekly inoculation rates were 4.95 and 5.05 in 2001 and 2002 respectively, while the seasonal (6-month) rates were 128.7 in 2001 and 131.3 in 2002 (Table 3).

Discussion

The apparently low number of anophelines per house was associated with the sampling method that yielded the residual resting fauna of females that had fed the previous night in the room. The increase in weekly human biting rate in 2002 was probably related to the sampling period, August/September, mid-rainy season. Awolola *et al*[22] had also observed that the peak biting by anophelines was during the mid-rainy season in coastal Lagos, south-western

Table 3. Anthropophilic, sporozoite and entomologic inoculation rates of *An. gambiae* s.l. at Igbo-Ora

Variables	Sampling period					
	2001			2002		
	June	July	Total	August	September	Total
Number of tenants at risk	59	139	198	75	40	115
Total number of *Anopheles*	136	354	490	323	176	499
Number fed + half-gravid *Anopheles*	52	128	180	124	60	184
Anopheles fed on humans	42	105	147	106	51	157
Anthropophilic rates (%)	81	82	82*	86	85	86*
Females harbouring CSP	9	24	33	22	10	32
Infection rate (%)	6.6	6.8	6.7	6.8	5.7	6.3
Weekly inoculation rate	2.78	7.12	4.95**	7.22	2.89	5.05**
Seasonal EIR (6-month)			128.7†			131.3

*Mean weekly anthropophilic rates; **Mean weekly inoculation rates; †Estimate of seasonal entomological inoculation rates.

Table 4. Anthropophilic rates of sibling species in *An. gambiae* s.l. at Igbo-Ora

Blood meal origin	*Anopheles gambiae* s.l.			
	An. arabiensis	*An. gambiae* s.s.	Non-identified	Total
Human	23 (7.7)	203 (67.9)	73 (24.4)	299 (83.99)
Other animals	3 (6)	31 (62)	16 (32)	50 (14.04)
Lost	1 (14.3)	5 (71.4)	1 (14.3)	7 (1.97)
Total	27 (7.58)	239 (67.13)	90 (25.28)	356 (100)
Anthropophilic rates (%)	88	87	81	86

Figures in parentheses indicate percentage.

Nigeria. Elissa *et al*[23] found that the biting rates of *Anopheles* vectors were 10 times higher in rural than in urban areas in Gabon. High cumulative human biting rates by the vectors *An. gambiae* s.s., *An. arabiensis* and *An. funestus* were one of the two principal reasons for the failure of the WHO-funded Garki project to control malaria by indoor residual spray in northern Nigeria (1969–76), the other reason was that the vector rested outdoors after blood feeding (exophily). Consequently, even a 90% reduction of vectorial capacity might have reduced malaria prevalence by only 25%[3].

Several factors contribute to the ability of a particular species to transmit malaria parasites, but the two most important are the mosquitoe's longevity and its propensity to feed on humans[24,25]. The mean weekly anthropophilic rates in 2001 and 2002 were high, both in *An. gambiae* s.s. and *An. arabiensis*; surprisingly, even those anophelines that could not be confirmed as one of the sibling species had high anthropophilic rates. The fact that the unidentified species had high anthropophilic rates highlights the concern of Touré *et al*[26] that thorough vector studies at specific sites were a pre-requisite to effective control measures. Since an individual mosquito must feed twice on humans before transmission, the probability of the mosquitoes feeding on human twice was high, in the range 0.81–0.88 in this study.

The EIR normally expresses the intensity of malaria parasite transmission and it is highly variable in Africa, with annual EIRs ranging from <1 to > 1000 infective bites per person per year[8]. It is the most favoured measure for assessing malaria endemicity and transmission intensity. EIR assessments may be particularly useful when estimating the effects of efforts to reduce human-vector contacts[27]. Weekly and seasonal inoculation rates obtained in this study were high compared to those recorded by Wanji *et al*[28] and Drakeley *et al*[29] in Cameroon and Tanzania respectively. The mean seasonal (6-month period) inoculation rates of 128.7 and 131.3 obtained in 2001 and 2002 respectively, were high compared to the mean annual entomologic rate of 167.7 obtained by Robert *et al*[30] from meta analyses of published data from rural areas in sub-Saharan Africa. Even at lowest EIRs, there were high rates of malaria prevalence and associated diseases[31–33]. The use of naked eye to read the ELISA plates was a limitation of the present study as this might have yielded lower anthropophilic and sporozoite rates. The actual EIRs were probably higher than those obtained. *An. gambiae* s.l. are therefore of major importance in the epidemiology of malaria at Igbo-Ora, contributing to the holoendemicity of the disease in this rural community[34,35].

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