

Resting Behavior of Malaria Vectors in Ghana and Its Implication on Vector Control.

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Research

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Abstract

Background: In Sub-Saharan Africa, there is widespread use of long-lasting insecticidal nets (LLINs) and Indoor residual spraying (IRS) to help control the density of malaria vectors and decrease the incidence of malaria in communities. An understanding of the interactions between increased insecticide use and resting behaviour patterns of malaria mosquitoes is important for an effective vector control programme. This study was carried out to investigate the resting behavior, host preference and infection with *Plasmodium falciparum* of malaria vectors in Ghana in the context of increasing insecticide resistance in malaria vectors in sub-saharan Africa.

Methods: Indoor and outdoor resting *Anopheline* mosquitoes were sampled during the dry and rainy seasons in five sites that were in 3 ecological landscapes [Sahel savannah (Kpalsogou, Pagaza, Libga), Coastal savannah (Anyakpor) and Forest (Konongo) zones] using pyrethrum spray catches (PSC), mechanical aspiration (Prokopack) for indoor collections, pit shelter and Prokopack for outdoor collections. PCR based molecular diagnostics were used to determine mosquito speciation, genotype for knockdown resistance mutations (L1014S and L1014F), G119S *Ace-1* mutation, specific host blood meal origins and sporozoite infection in field collected mosquitoes.

Results: *Anopheles gambiae* s. l. was the predominant species (89.95%, n = 1,718), followed by *An. rufipes* (8.48%, n=162), and *An. funestus* s. l. (1.57%, n = 30). Sibling species of the *Anopheles gambiae* revealed *An. coluzzii* accounted for 63% (95% CI: 57.10 – 68.91), followed by *An. gambiae* s. s [27% (95% CI: 21.66 – 32.55)], and *An. arabiensis* [9% (95% CI: 6.22 – 13.57)]. The mean resting density of *An. gambiae* s. l. was higher outdoors (79.63%; 1,368/1,718) than indoors (20.37%; 350/1,718) ($z = -4.815, p < 0.0001$). The *kdr* west L1014F and the *Ace-1* mutations were highest in indoor resting *An. coluzzii* and *An. gambiae* in the sahel-savannah sites compared to the forest and coastal savannah sites. Overall, the blood meal analyses revealed a large proportion of the malaria vectors preferred feeding on humans (70.2 %) than animals (29.8%) in all sites. The sporozoite rates was only detected in indoor resting *An. coluzzii* from the sahel savannah (5.0%) and forest (2.5%) zones.

Conclusion: The study reports high outdoor resting densities of *An. gambiae* and *An. coluzzii* with high *kdr* west mutation frequencies, and persistence of malaria transmission indoors despite the use of LLINs and IRS. Continuous monitoring of changes in resting behavior of mosquitoes and implementation of complementary malaria control interventions are needed to target outdoor resting *Anopheles* mosquitoes in Ghana.

Introduction

Malaria is a major public health problem in Africa and was responsible for an estimated 229 million episodes and 409 000 deaths in 2019. In Ghana, malaria is responsible for more than 5.5 million infections and 37 deaths for every 1,000 population (1, 2) despite tremendous efforts in the scale-up of vector control interventions particularly in the use of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (3, 4). These anti-vector control interventions previously led to a remarkable reduction of vector population (5-8) and malaria transmission (9, 10). However, there are reports of resurgence of malaria in many parts of sub-Saharan Africa (11, 12). Some of the challenges implicated in the resurgence is the emergence of insecticide resistance, behavioral modification (shift in the biting and resting behavior from indoor to outdoor) and changes in host species preferences from humans to animals (6, 13-15).

These challenges have arisen as an adaptation by the malaria vectors response to high use of insecticides for vector control. For instance, following the introduction of LLIN there has been reports of shift in biting behavior of *Anopheles gambiae* and *An. funestus* in Kenya (6, 16) and *An. funestus* in Benin and Senegal (17, 18). Whilst the long-term use of LLIN has increased the outdoor feeding proportion of *An. gambiae* and *An. melas* in Equatorial Guinea (13), in Tanzania, the long-term use of LLINs was reported to be associated with shifts in the outdoor resting rate of *An. gambiae*, *An. arabiensis* and *An. funestus* (14, 19). These behavioral changes however are not consistent with some countries reporting high indoor resting densities of *An. gambiae* and *An. funestus* despite long-term use of LLIN and IRS (20-22). Furthermore, the widespread insecticide resistance in malaria vector populations in Africa is a major threat to current malaria control programmes. Studies from Côte d'Ivoire (23), Togo (24, 25), Benin (26), Burkina Faso (27, 28), Cameroon (29, 30), and Kenya (31, 32) have reported high metabolic resistance and target site modification of insecticides in the malaria vector. In Ghana, the common target site resistance mechanisms in malaria vectors are the *Ace-1* mutation in acetylcholine esterase gene *G119S* that causes resistance to organophosphates and carbamates, and the voltage-gated sodium channel knockdown resistance (*Kdr*) which play a major role in resistance to pyrethroids are the most important mechanisms (33-35).

The primary malaria vectors in Ghana are the *Anopheles gambiae* sensu lato (*An. gambiae* s. s., *An. arabiensis*, *An. coluzzii* and *An. melas*) and *Anopheles funestus*. However, in view of the increasing concern about resurgence of malaria transmission in Africa (36, 37), there is a need to enhance control intervention strategies by having a better understanding of vectors resting and feeding behavior in their different specific settings and varying seasonal patterns. This is very crucial for the success of the current vector control tools and will provide a guide to improve efforts for the control of malaria in endemic regions.

The objective of this study was to investigate the resting behavior, species composition, insecticide resistance status, and *Plasmodium falciparum* infections of malaria vectors in three ecological settings of Ghana (the coastal savannah in the south, the forest in the middle, and the Sahel savannah in the north). These ecological zones have varying suitable weather and environmental conditions to sustain the propagation of *Anopheles* mosquitoes and malaria parasites (38). The coastal savannah and forest zone have a bimodal rainfall pattern, allowing for two peaks of malaria transmission while the Sahel zone has a unimodal rainfall pattern making malaria transmission seasonal. Information from this study could provide a better understanding of the impact of current malaria control programme effects on malaria vector population and their interaction on resting behavior.

Materials And Methods

Study sites

This study was carried out in five sites in three ecological landscapes of Ghana; Anyakpor in the coastal savannah zone, Dwease in the forest zone, and Kpalsogou, Libga and Pagaza in the Sahel savannah zone (Figure 1).

Anyakpor (5° 46'51.96 "N 0° 35'12.84 "E) is a village in the coastal savannah zone, about 5 km west of Ada Foah in Southern Ghana. The coastal savannah has a tropical savannah climate, with an average annual precipitation of 787 mm. Dwease (6° 32'3.05 "N 1° 14'42.22 "W) a village near Konongo, in the Asante-Akim Central district in the middle of Ghana was the site located in the forest zone. The forest zone has a tropical rainforest climate, with an average annual precipitation of 1399.5 mm. The climate in both the coastal savannah and forest area generally consists of a bimodal pattern of rainfall, with the long rainy season from March to June, and the short rainy season from October to November with mean annual temperature of 26.5°C.

Sites in the Sahel savannah ecological zone consisted of Kpalsogou (9° 33'45.2 "N 1° 01'54.6 "W), a village in the Kumbungu district of the northern region, Pagaza (9° 22'33.34 "N 0° 42'29.67 "W) in the Tamale metropolitan area and Libga (9° 35'32.26 "N 0° 50'48.8 "W), a village in the Savelugu-Nanton District. They have a unimodal rainfall pattern from May to November with a mean annual temperature of 28°C, which can get to a maximum of 42°C

Study Design

Mosquitoes were sampled during the rainy season in May for the sites in the coastal and forest zones (Anyakpor and Konongo respectively) and September for the sites in the Sahel savannah zones (Pagaza, Libga, and Kpalsogou) and the dry season in February to March at all the study sites in 2019. Sixteen houses were randomly selected in each study site and during each sampling night four houses were sampled for mosquitoes. Sampling was done over 4 days each during the dry and rainy seasons. The GPS coordinates of each site was determined and recorded.

Malaria vectors resting indoors were sampled using pyrethrum spray catches (PSCs) from 05.00 – 07.00 h (39). The Prokopack aspirator (John W Hock, Gainesville, FL, USA) was used to collect mosquitoes resting indoors and outdoors from 05.00 – 07.00h (40). For indoor collections, mosquitoes resting on the walls and under the roofs of houses or ceilings, under beds were systematically aspirated. Outdoor sampling points included kitchens, granaries, animal resting places and evening outdoor human resting points. Additionally, outdoor resting mosquitoes were collected from pit shelters constructed according to Muirhead-Thomson's method (41) within 20 m of each selected house. Resting mosquitoes in the cavities created in the pit shelter were collected from 06:00 to 07:00 h by using hand-held mouth aspirators.

Morphological Identification

All caught mosquitoes were counted and *Anopheline* mosquitoes were sorted morphologically according to the identification keys of Gillies and Coetzee (42). Sampled mosquitoes were further classified according to abdominal status as unfed, freshly fed, half-gravid and gravid. Mosquitoes from each collection method were stored in separately labeled vials with 95% ethanol. Samples were stored at the insectary of the Department of Medical Microbiology, University of Ghana Medical School, Accra, Ghana, until required for further processing.

Sibling Species Discrimination

Sibling species of the *An. gambiae* s. l. complexes were distinguished using the protocols of Scott et al. (43) and Fanello et al. (44). One leg from each mosquito serving as a DNA template was placed directly into the PCR master mix for Amplification.

Detection Of Sporozoites

The head and thorax of individual mosquito samples collected were used to detect the presence of *P. falciparum* sporozoites using sporozoite Polymerase Chain Reaction (PCR) as described by Echeverry *et al.* (45).

Detection Of Blood Meal Sources

The abdominal sections of blood-fed *Anopheles* mosquitoes were cut transversely between the thorax and the abdomen. Genomic DNA was extracted from mosquito abdomens using the ZR DNA MicroPrep™ kit (Zymo Research, CA) following the manufacturer's instructions. One universal reverse primer and five animal-specific forward primers (human, cow, goat, pig, and dog) were used for amplification of cytochrome b gene encoded in the mitochondrial genome to test for specific host blood meal origins using conventional PCR (46). Positive controls were included for each host during the PCR analyses and laboratory reared unfed *An. gambiae* was used as negative control.

Genotyping for kdr and Ace-1 mutations

To genotype for *kdr* mutations, DNA was extracted from mosquito legs using the ZR DNA MicroPrep™ kit (Zymo Research, CA) following the manufacturer's instructions. Standard PCR assays for L1014F *kdr* allele was used to test the presence of the *kdr* gene using a modification of the protocol by Ahadji-Dabla *et al.* (2019). In addition, the G119S mutation on the *Ace-1* gene was assessed using the PCR protocol described by Weill *et al.* [23].

Data Analysis

Resting densities of *Anopheles* mosquitoes was calculated as the number of female mosquitoes per trap/night for each trapping method. The Mann-Whitney U test was used to compare malaria vector density between indoor and outdoor locations. Chi-square was used to test the difference in seasonal abundance and malaria vector species composition between resting locations (indoor and outdoor).

Human blood index (HBI) was calculated as the proportion of blood-fed mosquito samples that had fed on humans to the total tested for blood meal origins. The sporozoite infection rate (IR) expressed as the proportion of mosquitoes positive for *Plasmodium* sporozoite was calculated by dividing the number of sporozoite positive mosquitoes by the total number of mosquitoes assayed.

The *Kdr*L1014F and *Ace-1* G119S mutation frequencies was calculated according to the following formula (47):

$$F = \frac{2(\text{HomozygoteResistant}) + \text{HeterozygoteResistant}}{2(\text{totalnumberofspecimenanalyzed})}$$

Sporozoite rates was calculated for examining pooled samples of mosquitoes using the Gu W. D. (48) formula below:

$$\text{varvecS} = \frac{\text{No. ofpositivepools}}{\text{No. ofpools} \times \text{Maximumpoolsize}} \times 100\%$$

Results

Indoor and Outdoor resting densities of female Anopheles mosquitoes

A total of 4,810 mosquitoes belonging to four genera were collected during the sampling period. Of these, 1,910 (39.71%) were *Anopheles* mosquitoes, 2,814 (58.50%) were *Culex*, 82 (1.70%) were *Aedes*, and 4 (0.08%) were *Mansonia*. The 1,910 *Anopheles* mosquitoes comprised of 1,718 (89.95%) *An. gambiae* s. l., 162 (8.48%) *An. rufipes*, and 30 (1.57%) *An. funestus*. Overall, 81.57% (1,558/1,910) of the *Anopheles* mosquitoes caught at the different sites were resting outdoors and 18.43% (352/1,910) were resting indoors ($z = -4.970$, $p < 0.0001$). The mean resting density of *An. gambiae* s. l. was higher outdoors (79.63%; 1,368/1,718) than indoors (20.37%; 350/1,718) ($z = -4.815$, $p < 0.0001$). More *An. funestus* were resting outdoors 93.33%; 28/30 compared to indoors (6.67%; 2/30) ($z = -2.039$, $p < 0.0001$). In addition, all (100%) of the *An. rufipes* caught in this study were resting outdoors.

In Kpalsogou, Pagaza and Libga (Sahel savannah zone), a total of 1,372, 104 and 76 female *Anopheles* mosquitoes were caught, whilst in Anyakpor (coastal savannah zone) and Konongo (forest zone) a total of 52 and 114 respectively were caught (Table 1). Out of the 1,372 *An. gambiae* s. l. collected in Kpalsogou, 13.41% [184/1372 (95% CI: 11.68 – 16.24)] were resting indoors and 86.59% [1,188/1,372(95% CI:84.64 – 88.32)] were resting outdoors (Table 1). All the *An. funestus* [100% (22/22)] and *An. rufipes* [100% (158/158)] were caught resting outdoors in Kpalsogou. In Pagaza and Libga, 7.7% [8/104 (95% CI: 3.62– 15.04)] and 50.0% [38/76 (95% CI: 39.03 – 60.10)] of *An. gambiae* were resting indoors, and 92.3% [96/104 (95% CI: 84.96– 96.38)] and 50.0% [38/76 (95% CI: 39.03 – 60.10)] were caught resting outdoors. More *Anopheles gambiae* s. l. were caught resting indoors in Anyakpor [84.6% (44/52) (95% CI: 71.37 – 92.66)] and Konongo [66.7% (76/114) (95% CI: 57.14 – 75.05)], than outdoors [15.4% (8/52) (95% CI: 7.34 – 28.63)] and 33.3% [38/114 (95% CI: 24.95 – 42.86)] respectively.

Table 1
Resting densities of mosquitoes collected from different sites in the three ecological zones in Ghana.

Site	Mosquito Species	Indoor No. (%)	Outdoor No. (%)	Total No.
Kpalsogou	<i>An. gambiae</i> s. l.	184 (13.41)	1188 (86.59)	1372
	<i>An. funestus</i> s. l.	0	22 (100)	22
	<i>An. rufipes</i>	0	158 (100)	158
Pagaza	<i>An. gambiae</i> s. l.	8 (7.69)	96 (92.31)	104
	<i>An. funestus</i> s. l.	0	0	0
	<i>An. rufipes</i>	0	0	0
Libga	<i>An. gambiae</i> s. l.	38(50)	38(50)	76
	<i>An. funestus</i> s. l.	2 (50)	2 (50)	4
	<i>An. rufipes</i>	0	0	0
Anyakpor	<i>An. gambiae</i> s. l.	44 (84.62)	8 (15.38)	52
	<i>An. funestus</i> s. l.	0	0	0
	<i>An. rufipes</i>	0	4 (100)	4
Konongo	<i>An. gambiae</i> s. l.	76 (66.67)	38 (33.33)	114
	<i>An. funestus</i> s. l.	0	4 (100)	4
	<i>An. rufipes</i>	0	0	0
Total	<i>An. gambiae</i> s. l.	350 (20.37)	1368 (79.63)	1718
	<i>An. funestus</i> s. l.	2 (6.67)	28 (93.33)	30
	<i>An. rufipes</i>	0	162 (100)	162

Seasonal Densities Of Mosquitoes From Different Sites

In the dry season, high numbers of *Anopheles* mosquitoes [1,214 (63.56%)] were sampled in the dry season than in the rainy season [696 (36.44%); ($z = -1.503$, $p = 0.1329$); Table 2]. In the Sahel savannah zone (Kpalsogou, Pagaza and Libga), a total of 1,054 (67.91%), 10 (9.62%), 32 (45.5%) vs. 498 (32.1), 48 (54.5%) and 94 (90.38%) female *Anopheles* mosquitoes were caught in the dry and rainy seasons. Whilst Konongo (the forest zone) high numbers of *Anopheles* were caught in the dry season [88.14%, (104/118)] compared to the rainy season [11.86%, (14/118)], in Anyakpor (the coastal savannah zone) there were more *Anopheles* collected in the rainy season [75%, (42/56)] than in the dry season [25%, (14/56)].

In all, the abundance of *Anopheles gambiae* s. l. was 62.28% [1,070/1,718 (95% CI: 59.94 – 64.57)] in the dry season and 37.72% [648/1718 (95% CI: 35.43 – 40.06)] in the rainy season (Table 2). *An. rufipes* were also more abundant in the dry season [88.89%, 144/162 (95% CI: 79.48 – 94.48)] than in the rainy season and [11.11%, 18/162 (95% CI: 6.70–17.24)]. *An. funestus* was only caught during the rainy season at Kpalsogou, Libga and Anyakpor sites. No *An. funestus* was caught in the dry season at any of the study sites.

Table 2
Total Mosquitoes collected from different sites in the three ecological zones in Ghana during the dry and rainy seasons.

Season	Location	<i>An. gambiae</i> s.l.	<i>An. funestus</i> s.l.	<i>An. rufipes</i>	Total
Dry	Kpalsogou	910	0	144	1054
	Pagaza	10	0	0	10
	Libga	32	0	0	32
	Anyakpor	14	0	0	14
	Konongo	104	0	0	104
	Subtotal (%)	1,070 (62.28)	0	144 (88.89)	1,214
Rainy	Kpalsogou	462	22	14	498
	Pagaza	94	0	0	94
	Libga	44	4	0	48
	Anyakpor	38	0	4	42
	Konongo	10	4	0	14
	Subtotal	648 (37.72)	30 (100)	18 (11.11)	696
	Total	1,718 (100)	30 (100)	162 (100)	1,910

Anopheles gambiae sibling species composition

A sub-sample of 538 *An. gambiae* s. l. from all study sites were analysed for the identification of their respective sibling species. Overall, *An. coluzzii* accounted for 63% (95% CI: 57.10 – 68.91), followed by *An. gambiae* s. s. hereafter (*An. gambiae*) [27% (95% CI: 21.66 – 32.55)], *An. arabiensis* [9% (95% CI: 6.22 – 13.57)] and *An. melas* [1% (95% CI: 0.13 – 3.00)].

In Kpalsogou, 12% (194/1362) of *An. gambiae* s. l. were analyzed and of these 72% [140/194 (95% CI: 61.99 – 50.56)] were *An. coluzzii*, 17% [32/194 (95% CI: 10.01 – 25.71)] were *An. gambiae* and 11% [22/194 (95% CI: 6.07 – 19.79)] were *An. arabiensis*. In Pagaza, out of the 104 *An. gambiae* s. l. analyzed, 67% [70/104 (95% CI: 52.78 – 79.28)] were *An. coluzzii*, 25% [26/104 (95% CI: 14.48 – 39.23)] were *An. gambiae* s. s. and 8% [8/104 (95% CI: 2.49 – 19.40)] were *An. arabiensis*. Out of the 74 *An. gambiae* s. l. analyzed in Libga, 54% [40/74 (95% CI: 37.13 – 70.15)] were *An. gambiae*, 27% [20/74 (95% CI: 14.37 – 44.39)] were *An. arabiensis* and 19% [14/74 (95% CI: 8.56 – 35.71)] were *An. coluzzii*.

In Anyakpor (the coastal zone), out of the 52 *An. gambiae* s. l. analyzed for sibling species, 84% [44/52 (95% CI: 64.27 – 94.95)] were *An. coluzzii*, and the rest were *An. gambiae* [8% (2/26); 95% CI: 1.34 – 26.60] and *An. melas* [8% (2/26); 95% CI: 0.12 – 2.95]. In contrast, the forest zone (Konongo) had only *An. coluzzii* [63% (72/114); 95% CI: 49.30 – 75.24] and *An. gambiae* [37% (42/114); 95% CI: 24.76 – 50.70].

The seasonal composition of the two malaria vectors (*An. coluzzii* and *An. gambiae*) varied at the different sites; in the Sahel savannah zone; *An. coluzzii* was higher outdoors (41.2% vs. 4.4%) than indoors (26.5% vs. 1.5%) for Kpalsogou and Pagaza in the dry season (Figure 2A). In contrast, Libga had more *An. gambiae* dominating outdoors (7.4%) than indoors (4.4%). In Konongo, high densities of *An. coluzzii* and *An. gambiae* was detected indoors (39.7% vs. 22.1%) compared to outdoors (5.8% vs. 2.9%).

In the rainy season, *An. arabiensis*, *An. coluzzii* and *An. gambiae* were caught both indoors and outdoors in Kpalsogou, Pagaza, and Libga (Figure 2B). High densities of *An. coluzzii* were caught indoors (25%) than outdoors (6.7%) in Anyakpor. However, in Konongo the densities of *An. coluzzii* and *An. gambiae* were slightly higher indoors (5.1% vs. 3.1%) than outdoors (2.7% vs. 2.7%).

Kdr resistance mutations between indoor resting and outdoor resting *Anopheles gambiae*

A total of 538 *Anopheles gambiae* s.l. samples were genotyped for the presence of L1014S, L1014F and G119S *Ace-1* mutations. The L1014F *kdr* allele was identified in 100% (538) of the samples, with the majority of mosquitoes being homozygous for the *kdr* allele (70.6%; 380/538). Overall, the *kdr* mutation frequency at Sahel savannah sites (Kpalsogou, Pagaza and Libga) was higher in mosquitoes resting indoors (0.90) than outdoors (0.84). In the forest zone (Konongo), a higher *kdr* frequency was detected in mosquitoes resting outdoors (0.9) than indoors (0.79). In the coastal zone (Anyakpor), a similar *kdr* frequency was detected in mosquitoes collected indoors (0.88) and outdoors (0.83). No *Kdr* L1014S mutation was identified in this study.

An. arabiensis collected from outdoors at Pagaza and Libga had higher *kdr* mutations (0.75 vs. 0.86) compared to indoors (0 vs. 0.83). However, in Kpalsogou *An. arabiensis* had higher *kdr* mutations indoor (1) than outdoor (0.88) (Table 3). Indoor resting *An. coluzzii* from Kpalsogou (0.90), and Pagaza (0.90) had a higher *kdr* mutation frequency than outdoor resting *An. coluzzii* (0.84 vs. 0.70). *An. gambiae* caught outdoor had a higher *Kdr* mutation frequency in Pagaza (0.88) and Libga (0.86) than indoor resting *An. gambiae* (0.5 vs. 0.83).

G119S Ace-1 mutations in indoor and outdoor malaria vectors

The G119S *Ace-1* mutations was detected in 79.9% (215/538) of the mosquitoes tested (Table 4). All the mosquitoes with the resistant allele were heterozygous for this mutation. Overall, similar *Ace-1* mutations frequencies was detected in indoor and outdoor resting mosquitoes in Sahel savannah zone (0.76 vs. 0.78). In the forest zone, *Ace-1* mutations was slightly higher in mosquitoes resting indoors (0.87) than outdoors (0.80), but higher in mosquitoes resting outdoor (1) than indoor (0.76) in the coastal zone.

The frequency of *Ace-1* mutation was similar for *An. coluzzii* collected indoors and outdoors in Kpalsogou (0.38 vs. 0.39) and Libga (0.5 vs. 0.5). However, high *Ace-1* mutation was detected in outdoor (0.42) than indoor (0.35) resting *An. coluzzii* in Pagaza (Table 4). In Kpalsogou, Pagaza, and Libga (Sahel savannah sites), the *Ace-1* mutation was higher for *An. gambiae*. resting indoors (0.5, 0.5, 0.38) than outdoors (0.38, 0.42, 0.36)

Table 3
Frequency of *Kdr*L1014F mutation in the different species of *Anopheles gambiae* complex.

Site	Location	<i>An. arabiensis</i>				<i>An. coluzzii</i>				<i>An. gambiae s. s.</i>				<i>An. melas</i>			
		No. tested	RS	RR	F(<i>Kdr</i>)	No. tested	RS	RR	F(<i>Kdr</i>)	No. tested	RS	RR	F(<i>Kdr</i>)	No. tested	RS	RR	F(<i>Kdr</i>)
Kpalsogou	Indoor	6	0	6	1	58	12	46	0.90	8	0	8	1				
	Outdoor	16	4	12	0.88	82	26	56	0.84	24	4	20	0.92				
	Total	22				140				32							
Pagaza	Indoor	0	0	0	0	20	4	16	0.9	2	2	0	0.5				
	Outdoor	8	4	4	0.75	50	30	20	0.7	24	6	18	0.88				
	Total	8				70				26							
Libga	Indoor	6	2	4	0.83	6	0	6	1	18	6	12	0.83				
	Outdoor	14	4	10	0.86	8	0	8	1	22	2	20	0.95				
	Total	20				14				40							
Anyakpor	Indoor					32	4	28	0.94	0	0	0	0	2	2	0	0.5
	Outdoor					12	0	12	1	4	2	2	0.75	2	0	2	1
	Total					44				4				4			
Konongo	Indoor					60	24	36	0.80	34	16	18	0.76				
	Outdoor					12	0	12	1.00	8	4	4	0.75				
	Total					72				42							

Abbreviations: RR homozygote resistant, RS heterozygote resistant, F frequency

Table 4
Frequencies of *Ace-1* mutation of *An. gambiae* s. l. at the different sites in the three ecological zones in Ghana.

Site	Species			Genotype				
		Total No. Tested	No. Tested	Indoor	GS	GG	SS	F (<i>ace-1</i>)
					n (%)	n (%)		
Kpalsogou	<i>An. arabiensis</i>	22	6	Indoor	4(18.2)	2 (9.1)	0	0.33
			16	Outdoor	6(27.3)	10 (45.5)	0	0.19
	<i>An. coluzzii</i>	140	58	Indoor	44 (31.4)	14 (10)	0	0.38
			82	Outdoor	64 (45.7)	18(12.9)	0	0.39
	<i>An. gambiae</i> s. s.	32	8	Indoor	4 (25)	0	0	0.50
			24	Outdoor	18(56.25)	6 (18.75)	0	0.38
Pagaza	<i>An. arabiensis</i>	8	0	Indoor	0	0	0	0.00
			8	Outdoor	8(100)	0	0	0.50
	<i>An. coluzzii</i>	70	20	Indoor	14(20)	6 (8.6)	0	0.35
			50	Outdoor	42(60)	8 (11.4)	0	0.42
	<i>An. gambiae</i> s. s.	26	2	Indoor	2(7.7)	0	0	0.50
			24	Outdoor	20 (76.9)	4 (15.4)	0	0.42
Libga	<i>An. arabiensis</i>	20	6	Indoor	2 (20)	4(20)	0	0.17
			14	Outdoor	12(60)	2 (10)	0	0.43
	<i>An. coluzzii</i>	14	6	Indoor	6(42.9)	0	0	0.50
			8	Outdoor	8 (57.1)	0	0	0.50
	<i>An. gambiae</i> s. s.	40	18	Indoor	14 (35)	4 (10)	0	0.39
			22	Outdoor	16 (40)	6 (15)	0	0.36
Anyakpor	<i>An. coluzzii</i>	44	32	Indoor	24 (54.5)	8 (18.2)	0	0.38
			12	Outdoor	12 (27.3)	0	0	0.50
	<i>An. gambiae</i> s. s.	4	0	Indoor	0	0	0	0.00
			4	Outdoor	4(100)	0	0	0.50
	<i>An. melas</i>	4	2	Indoor	2(50)	0	0	0.50
			2	Outdoor	2 (50)	0	0	0.50
Konongo	<i>An. coluzzii</i>	72	60	Indoor	52 (72.2)	8 (11.1)	0	0.43
			12	Outdoor	10(13.9)	2 (2.8)	0	0.42
	<i>An. gambiae</i> s. s.	42	34	Indoor	30(71.4)	4(9.5)	0	0.44
			8	Outdoor	6(14.3)	2 (4.8)	0	0.38
Grand total		538			215 (79.9%)	54 (20.1)	0	

Abbreviations: GS heterozygote resistant, GG homozygote susceptible, SS homozygote resistant

Blood meal sources

The 362 *An. gambiae* s. l. blood fed mosquito specimen analyzed for blood meal origins revealed a total human blood index (HBI) of 70.2% (254/362) across all sites. Overall, indoor resting mosquitoes had a slightly higher [73.1%, (136/186)] HBI than outdoor [67%, (118/176)] resting ones. In the savannah zone (Kpalsogou, Libga and Pagaza), the overall HBI of mosquitoes resting outdoors was higher [67.5%, (52/77)] than indoor resting mosquitoes [32.5% (25/77)]. In Kpalsogou, the human blood index (HBI) for *An. arabiensis*, *An. coluzzii* and *An. gambiae* caught resting indoor and outdoor was 100%, 68.4% and 50% vs. 40%, 70% and 54.5% respectively (Table 5). Only indoor and outdoor resting *An. coluzzii* were positive for human blood with an HBI of 66.7% and 100% respectively in the coastal zone (Anyakpor) (Table 5). In Konongo (Forest zone) the overall HBI was similar for indoor (84.7%) and outdoor (85.7%) resting mosquitoes. The HBI for *An. coluzzii*, and *An. gambiae* caught resting indoor and outdoor was 92.3% and 75% vs. 88.2% and 100% at the Konongo site.

Table 5: Blood meal origins of *Anopheles* mosquitoes collected from indoor and outdoor in three ecological zones in Ghana.

Site	Blood-meal origins	<i>An. arabiensis</i>		<i>An. coluzzii</i>		<i>An. gambiae s. s.</i>	
		Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
Kpalsogou	Number tested	2	10	38	60	4	22
	Human	2 (100)	4 (40)	26 (68.4)	42 (70)	2 (50)	12 (54.5)
	Goat	0	6 (60)	10 (26.5)	10 (16.7)	2 (50)	10 (45.5)
	Cow	0	0	2 (5.3)	4 (6.7)	0	0
	Dog	0	0	0	4 (6.7)	0	0
	HBI	100	40	68.4	70	50	54.5
	BBI	0	0	5.3	6.7	0	0
Pagaza	Number tested	0	6	16	8	2	8
	Human	0	4 (66.7)	8 (50)	14 (77.8)	2 (100)	6(75)
	Goat	0	2 (33.3)	8 (50)	4 (22.2)	0	2 (25)
	HBI	0	66.7	50	77.8	100	75
	BBI	0	0	0	0	0	0
Libga	Number tested	6	10	2	8	12	16
	Human	4 (66.7)	8(80)	2 (100)	8 (100)	4 (33.3)	6 (37.5)
	Goat	2 (33.3)	2 (20)	0	0	8 (66.7)	8 (50.0)
	Dog	0	0	0	0	0	2 (12.5)
	HBI	66.7	80	100	100	33.3	37.5
	BBI	0	0	0	0	0	00
	Number tested			12	1	0	2
	Human			8 (66.7)	2 (100)	0	0
Anyakpor	Dog			4 (33.3)	0	0	2 (100)
	HBI			66.7	100	0	0
	BBI			0	0	0	0
Konongo	Number tested			52	8	34	6
	Human			48 (92.3)	6 (75)	30 (88.2)	6 (100)
	Goat			4 (7.7)	0	4(11.8)	0
	Pig			0	2 (25)	0	0
	Un-identified			3	0	0	0
	HBI			92.3	75	88.2	100
	BBI			0	0	0	0

Abbreviations: HBI, Human blood index; BBI, bovine blood index

Sporozoites infection rates

A total of 68 pools of head and thoraces of *Anopheline* mosquitoes (20 Kpalsogou, 8 Pagaza, and 16 Libga, 8 Anyakpor, 16 Konongo) were constituted and tested for *Plasmodium falciparum* Circumsporozoite protein (CSP). Four pools (2 pools from Kpalsogou and 2 pools from Konongo) tested positive for *Plasmodium falciparum* Circumsporozoite protein giving an overall infection rate of 0.6%. Overall, the sporozoite rate was higher for indoor resting mosquitoes from Kpalsogou (5.0%) and Konongo (2.5%) (Table 6). None of the mosquitoes collected outdoors in Kpalsogou, Pagaza, Libga, Anyakpor

and Konongo was positive for *P. falciparum* CSP. CSP was detected in only *An. coluzzii* (2.5%) collected in the rainy season in Kpalsogou and in the dry season (2.0%) in Konongo.

Table 6: Sporozoite infections in *Anopheles gambiae* s. l from three ecological zones in Ghana.

	Location	<i>An. arabiensis</i>		<i>An. coluzzii</i>		<i>An. gambiae</i> s. s.	
		Pools tested	Pf +ve	Pools. tested	Pf +ve	Pools	Pf +ve
Kpalsogou	Indoor	-	-	4	2 (5%)	-	-
	Outdoor	2	0	8	0	6	0
Pagaza	Indoor	-	-	-	-	4	0
	Outdoor	2	0	2	0	-	-
Libga	Indoor			4	0		
	Outdoor			12	0		
Anyakpor	Indoor			4	0	-	-
	Outdoor			0	0	2	0
Konongo	Indoor			8	2(2.5%)	6	0
	Outdoor			2	0	2	0

Discussion

Behavioral diversification in vector population threatens to counteract vector control strategies in areas with widespread use of LLINs and IRS (6, 7, 13-15, 49). This study investigated the behavioral response of malaria vectors for resting, feeding choices and infection rates in the context of increasing insecticide resistance in malaria vectors. Overall, this study revealed high outdoor resting densities of malaria vectors but with lower infection rates. There were high numbers of mosquitoes with insecticide resistance mutations resting indoors compared to outdoors at the different sites in the three ecological zones in Ghana.

Anopheles coluzzii and *An. gambiae* from Kpalsogou, Libga and Pagaza (Sahel savannah zone) showed increased outdoor resting tendency in both the rainy and dry season despite high coverage and usage of LLINs and IRS (50). This could drive malaria transmission higher outdoors since these main malaria vectors can bite unprotected humans outdoors and also rest outdoors to avoid contact with the insecticides being used indoors (51, 52). This behavioral change in malaria vector population is detrimental to vector control [13, 14], which mainly target vectors resting indoors. Such variation in the relative frequency and behavior of this two main malaria vectors has been reported in studies from Equatorial Guinea (13), Tanzania (14, 19), but, in contrast to studies from Kenya (20, 49) which reported high indoor resting densities of *An. gambiae* and *An. coluzzii*.

In this study, it was found that the frequency of *kdr* west mutation L1014F and the *Ace*-1 mutation were of higher frequency in indoor resting *An. coluzzii* and *An. gambiae* in the sahel-savannah sites compared to the forest and coastal savannah sites. This may be because the interventions indoors are not able to kill the mosquitoes with higher *kdr* mutations and mosquitoes with less mutations have resorted to feed and rest outdoors to avoid contact with insecticides that are indoors (31, 67-69). This study is in conformity to previous studies in Ghana (33, 34) which reported similar occurrence of *Kdr* L1014F frequencies in *An. coluzzii*. This study is also in conformity to previous studies in Ghana (33, 34) which reported no *Kdr*-east allele. However, in contrast to this study, *Kdr*-east allele (1014S) have been reported in Burkina Faso in *An. coluzzii*, *An. gambiae*, and *An. arabiensis* (53), and in both *An. coluzzii* and *An. gambiae* in Togo (25).

The blood meal analyses revealed a large proportion of the malaria vectors preferred feeding on humans than animals in almost all sites. This human-host choice and higher outdoor resting proportions of *An. gambiae* and *An. coluzzii* poses a great concern in malaria elimination efforts due their efficiency in transmitting malaria. This study findings is in agreement with studies by Orsborne *et al.* (54) in the coastal area of Ghana, which reported that blood-fed mosquitoes caught indoors had higher HBI and lower BBI than those caught outdoors.

Sporozoite infection was only found in indoor resting malaria vectors collected during the wet season from Kpalsogou and dry season from Konongo. This might mean that malaria transmission is higher indoors than outdoors despite the deployment of LLINs in all sites and IRS in Kpalsogou (55). In

contrast, studies from Kenya, Burkina Faso have shown higher sporozoite infection in *An. gambiae* and *An. coluzzii* resting outdoors (20, 49, 56), and in *An. gambiae* sampled from Northern Ghana (7).

Conclusions

The study revealed high densities of *An. coluzzii* and *An. gambiae* resting outdoors with low genotypic resistance compared to indoor resting mosquitoes that may be triggered by current insecticide-based indoor intervention. Such behavioral change can promote outdoor transmission since current vector strategies mainly target indoor resting malaria vectors. There is a need for further screening of other resistance mutations in this population for better resistance management strategies. Therefore, continuous monitoring of vector behavior in surveillance programmes is recommended and complementary malaria control interventions are needed to control outdoor resting mosquitoes.

List Of Abbreviations

BBI, bovine blood index; CSP, circumsporozoite protein; GS, heterozygote resistant; GG, homozygote susceptible; HBI, human blood index; IRS, indoor residual spray; KDR, Knockdown resistant gene; LLINs, long-lasting insecticidal nets; PCR, polymerase chain reaction; PSC, pyrethrum spray catch; SS, homozygote resistant.

Declarations

Ethics approval

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AOF designed, performed the field and laboratory work, analyzed data and drafted the manuscript. YAA conceived and supervised the study, analyzed data and revised the manuscript. SKA and IAH supervised the study and revised the manuscript. IAH, SBD, ARM and IKS performed field and laboratory experiments.

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Figures

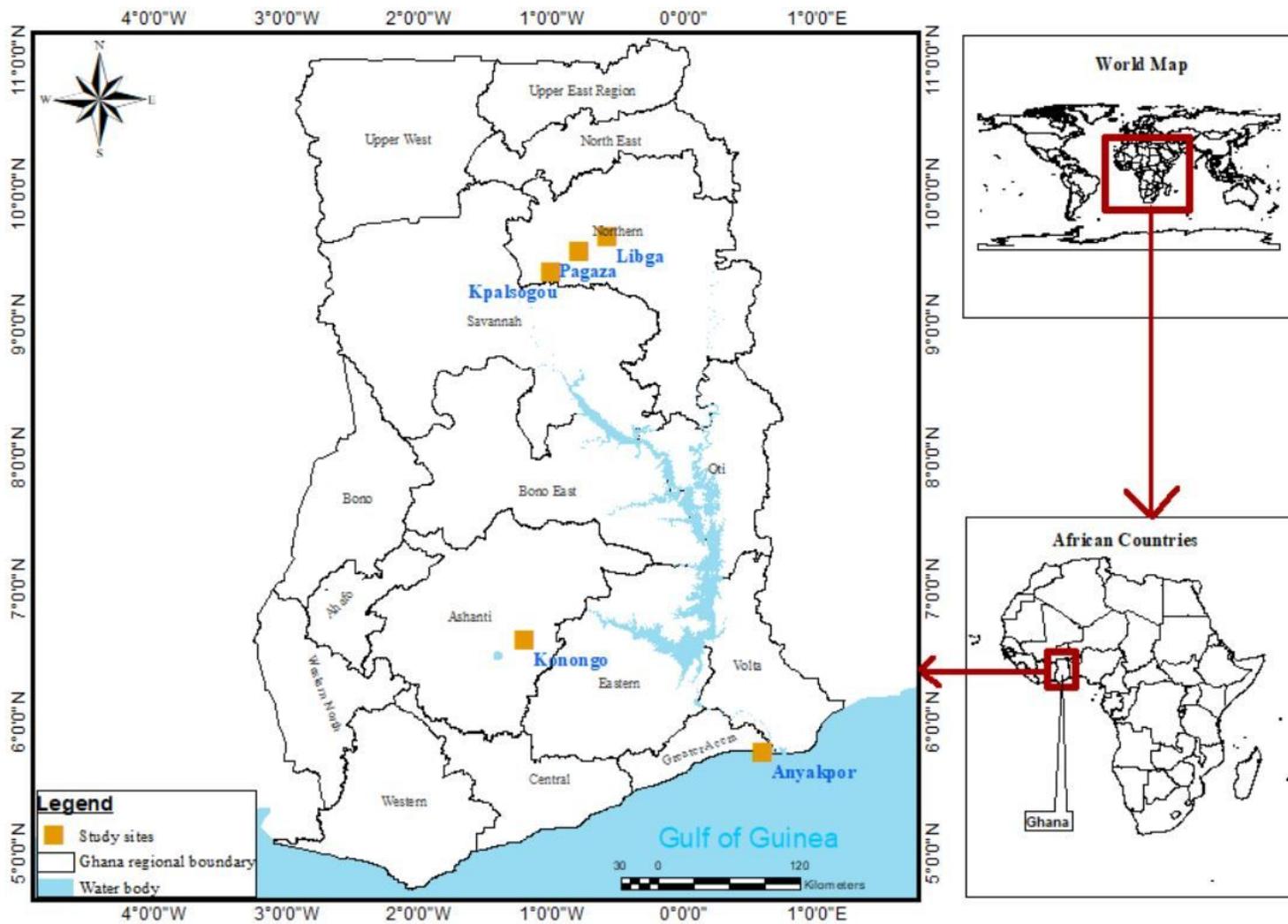


Figure 1

Map of Ghana showing the different study sites

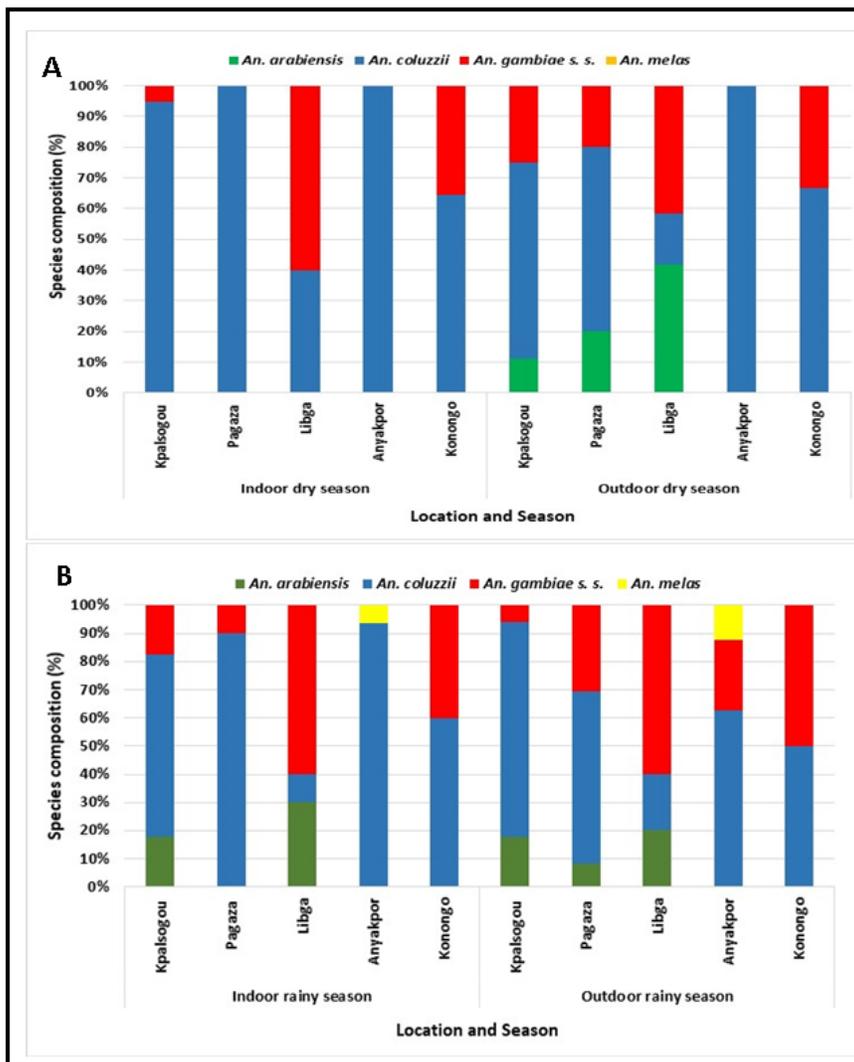


Figure 2 Seasonal composition of *Anopheles* species resting indoor and outdoor in **A**: Dry season and **B**: Rainy season in different sites at the three main ecological zones of Ghana.

Supplementary Files

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