Parasites
&Vectors

Implications of low-density microfilariae carriers in *Anopheles* transmission areas: molecular forms of *Anopheles gambiae* and *Anopheles funestus* populations in perspective

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Abstract

Background: Previous studies have shown a general reduction in annual transmission potential (ATP) of *Anopheles* species after mass drug administration (MDA) in lymphatic filariasis endemic communities. Whereas results obtained from a monitoring programme after three years of MDA revealed a decrease in ATP of *Anopheles funestus* this was not the same for *An. gambie* s.s. in Ghana. In this study, the ability of these vectors in transmitting *Wuchereria bancrofti* in nine lymphatic filariasis endemic communities in Gomoa District of Ghana after four rounds of MDA with ivermectin and albendazole was investigated.

Methods: After mass screening of inhabitants in these communities, twelve consenting volunteers with different intensities of microfilariae (mf) slept under partly opened mosquito nets as sources of mf blood meal. Hourly collection of mosquitoes and finger-pricked blood were taken from 21.00 to 06.00 hours the following day. For each hour, half of the mosquitoes collected were immediately killed and dissected for mf. The remaining half were maintained up to 13 days for parasite maturation. Parasitaemia and infection rates in the mosquitoes were determined by microscopy. The mosquitoes were identified by microscopy and molecular techniques.

Results: A total of 1,083 participants were screened and the overall parasite prevalence was 1.6% with mf intensities ranging from 0 to 59 per 100 μl and geometric mean intensity of 1.1 mf per ml of blood. Of the 564 mosquitoes collected, 350 (62.1%) were *Anopheles* spp., from which 310 (88.6%) were *An. funestus* and 32 (9.1%) *An. gambiae*. Six anopheline mosquitoes (1.7%) were found infected with L_1, but no larva was observed in any of the mosquitoes maintained up to 13 days. Molecular studies showed all *An. gambiae* s.l. to be *An. gambiae* s.s., of which 21 (70%) were of the M molecular form.

Conclusion: At low-level parasitaemia after 4 rounds of MDA, there was no recovery of infective stage larvae of *W. bancrofti* in *An. funestus* s.l. as well as M and S forms of *An. gambiae*.

Keywords: Mass drug administration, Low-density microfilariae carriers, *Wuchereria bancrofti*, *Anopheles gambiae*, *Anopheles funestus*

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Background

Wuchereria bancrofti is one of the three filarial worms responsible for about 90% of all lymphatic filariasis (LF) cases in the world [1]. These parasites are transmitted through the bite of infective mosquitoes of various genera. A competent vector is one that is capable of ingesting microfilariae (mf) from an infected human, supporting their development to the infective stage larvae (L_3) and subsequently transmitting them to other uninfected persons. Depending on the vector species, its region of origin and the parasite density ingested, this ability to sustain the maturation and transmission of LF may be enhanced or restricted [2,3]. Low density mf is defined as the density of circulating mf in a specified blood compartment that cannot be detected in a significant number of instances when commonly used blood sampling techniques are applied in epidemiological studies; thus 4 mf per 20 μl (200 mf per ml) [4]. Zhang *et al.* [5] in their study reported that between 1.55 and 2.23% prevalence, there was a threshold provided no individual had an mf density of more than 12 mf per 60 μl of blood.

The Global Programme to Eliminate Lymphatic Filariasis (GPELF) was launched in 2000, with the main goal of halting transmission and reducing disability through annual mass drug administration (MDA) to all persons at risk of infection, particularly if the vectors are *Anopheles* species [6]. The strategy relies on the assumption that if the mf reservoir in the human host is reduced below a certain threshold, transmission of *W. bancrofti* by anopheline vectors could be interrupted [7]. This is due to the observation that even though *Anopheles* mosquitoes yield more infective stage larvae than *Culex* species, the latter is more efficient at ingesting and developing low-density mf (limitation) than the former [4]. Thus *Anopheles* mosquitoes are presumed to be efficient vectors of LF when the parasite density in the human population is high, a phenomenon known as "facilitation" [4]. This observation has been the source for the heightened interest in the advocacy for the possible elimination of anopheline-transmitted filariasis; however, a study has observed "facilitation" in *An. gambiae* s.s. and *An. arabiensis* but not in *An. melas* in Gambia or *An. merus* in Tanzania [4]. Additional health benefits of MDA targeting LF is the reduction in soil transmitted helminths and scabies [8].

A study in the Bongo district of northern Ghana [9] indicates a plausible "limitation" in *An. gambiae* s.l. and/or *An. funestus* in the transmission of the parasite contrary to other reports [4]. Results from a study in the Gomoa district of southern Ghana also indicated that although transmission potential by *An. funestus* has decreased significantly after mass chemotherapy with ivermectin and albendazole, there appears to be no change in *An. gambiae* s.s. in the area (Boakye DA, unpublished data). This suggests that probably not all anophelines exhibit facilitation in their transmission of LF. This work was therefore conducted to determine the roles of these two *Anopheles* species in the transmission of low level *W. bancrofti* human mf, since this information is fundamental to the success of GPELF.

Methods

Study sites

Nine LF endemic communities in the Gomoa district of Ghana (between Latitude 5° 24' - 35'N and Longitude 0° 25' - 36'W) were selected based on available data on the disease epidemiology in the population and vector species distribution [10-12]. These are Amanful, Ayesuano, Dago, Fawomanye, Hwida, Kyiren, Mampong, Obiri and Okyereko. The district lies in the coastal savannah zone of Ghana and is located 50 km west of Accra, the capital city of Ghana. Average annual rainfall ranges between 760 and 1000 mm, whilst mean annual temperature ranges between 26 and 30°C. The main occupations of the inhabitants are farming and fishing for those living near the shores of the Atlantic Ocean.

Mass screening for microfilariae in the communities

This study was conducted from April to June 2004, the fourth year of MDA with ivermectin and albendazole in these communities. The areas also form part of an on-going annual longitudinal community-based intervention study. Human participation and the mosquito collection were done by cluster sampling method. Mass screening of the study population for mf was done by collecting 100 μl finger-pricked blood from each individual into heparinised capillary tubes and immediately mixing with 900 μl 3% acetic acid. Quantification of parasitaemia used the Sedgwick-Rafter counting chamber method with the compound microscope set at ×100 magnification [13].

Mosquito collection, maintenance and dissection

After consenting to participate, twelve adult volunteers with varying mf levels slept under partially opened mosquito nets hung over beds in their rooms. At the mid-point of each collection hour, finger-pricked blood was taken and mf density estimated using the same procedure described above. Mosquitoes trapped in the nets were collected each hour from 21.00 hours to 06.00 hours on the next day using an aspirator. About half the number of mosquitoes collected were killed immediately and dissected for ingested mf. The remaining mosquitoes were fed on 10% sugar solution and maintained for up to 13 days in paper-cups at 26-28×C, relative humidity 70-80% and 12-hour photoperiod in the insectary [14]. Mosquitoes that died before day 13 were dissected for developing stages of *W. bancrofti*, whilst those that survived until the last day were dissected for the presence of infective stage L_3 larvae of the parasite.

PCR identification of *Anopheles* species

Molecular identifications of *An. gambiae*, *An. funestus* and *W. bancrofti* were conducted using already established methods [15-17]. For the vector species identification, genomic DNA was extracted from the carcasses of mosquitoes after homogenisation with sterile Konte's plastic pestles in 100 µl bender buffer. The homogenate was then incubated at 65°C for 30 min, followed by the addition of 125 µl of phenol. The Centrifuge 5415 C (Eppendorf) was used in all spinning of samples, unless otherwise stated. The mixture was vortexed and spun at 14,000 rpm for 10 min. The supernatant was transferred into a fresh tube and 250 µl of pre-chilled absolute ethanol and 10 µl of 8 M potassium acetate were added. This was incubated at -40°C for an hour, spun at 10,000 rpm for 10 min and supernatant poured off. The pellet was then rinsed with 200 µl of 70% ethanol, spun at 10,000 rpm for 5 min, and supernatant poured off. The pellet was dried and re-dissolved in 50 µl TE + RNAse and then kept at 4°C until ready for PCR (Table 1). Each PCR reaction mixture of 25 µl contained 1× PCR buffer (Sigma, USA), 200 µM each of the four deoxyribonucleotide triphosphates, 10 µM each of the oligonucleotide primers (Table 1), and 0.125 units of *Taq* Polymerase enzyme (Sigma, USA). A microliter of the genomic DNA was used as template for the amplification reaction. *Anopheles gambiae* s.s. were further identified and differentiated into the M and S molecular forms by enzymatic restriction of the PCR product as described by Fanello *et al.* [18]. This was done by amplification of 1.3 kb rDNA followed by restriction fragment length polymorphism (RFLP) with restriction enzyme *Hha* I (Sigma-Aldrich, USA).

After gene amplification and digestion, the PCR products were electrophoresed separately in 2% agarose gel. The gel was prepared by adding TAE buffer to the powder, which was placed in a microwave oven (230 V, 50 Hz, 2660 W, 12.0A) for 1 minute to dissolve the solute, and then stained with 0.5 µg/ml Ethidium Bromide. For the electrophoresis, 8 µl of each sample was added to 1 µl of orange G (5X) gel loading dye after placing the solidified gel in 1X TAE buffer in a mini gel system (BIORAD USA). One hundred volts of electric current was passed through it for an hour and the gel photographed over a UV transilluminator (UPC, USA) at short wavelength using a Polaroid camera and film type 667 (Polaroid, USA). The sizes of the PCR products were estimated by comparison with the mobility of a 100 base pair molecular weight size marker (Sigma).

Molecular identification of *Wuchereria bancrofti* larvae in mosquito vectors

After the carcass of infected mosquito was scrapped into 1.5 ml eppendorf tubes, DNeasy Tissue Kit (QIAGEN Inc., USA) was used in the extraction of the parasite's genomic DNA from animal tissues following the manufacturer's recommended protocol. After the DNA extraction, aliquots of 5 µl of the filarial DNA extract from the mosquitoes were used as templates for the amplification reaction. The PCR assay was performed using two published specific oligonucleotide primers, NV-1 and NV-2 [17]. The PCR products were electrophoresed in 2% agarose gel as described in the previous section.

Table 1 Oligonucleotide primer sequences and PCR reaction conditions for species identification

Primer for species ID	Sequence 5' ⇒ 3'	PCR product size (bp)	PCR conditions
***Anopheles gambiae* s.l. species**			
Universal primer	GTGTGCCCCTTCCTCGATGT	468	93°C 3' followed by 35 cycles (93°C 30"; 50°C 30"; 72°C 1'); 93°C 30"; 50°C 30"; 72°C 10'
Anopheles gambiae s.s.	CTGGTTTGGTCGGCACGTTT	390	
Anopheles merus/melax	TGACCAACCCACTCCCTTGA	464	
Anopheles arabiensis	AAGTGTCCTTCTCCATCCTA	315	
Anopheles quadrianulatus	CAGACCAAGATGGTTAGTAT	153	
***Anopheles funestus* s.l. species**			
Universal primer	TGTGAACTGCAGGACACAT		30 cycles (94°C 30"; 40°C 30"; 72°C 30"); 72°C 10'
Anopheles funestus s.s.	GCATCGATGGGTTAATCATG	460	
Anopheles vaneedeni	TGTCGACTTGGTAGCCGAAC	555	
Anopheles rivulorum	CAAGCCGTTCGACCCTGATT	400	
Anopheles parensis	TGCGGTCCCAAGCTAGGTTC	235	
Anopheles leesoni	TACACGGGCGCCATGTAGTT	146	
Wuchereria bancrofti			
NV-1	CGTGATGGCATCAAAGTAGCG	188	94°C 3'; followed by 35 cycles (94°C 1'; 55°C 1'; 72°C 2'); 94°C 1'; 55°C 1'; 72°C 10"
NV-2	CCCTCACTTACCATAAGACAAC	188	

Table 2 Prevalence of mf and the geometric mean intensity in the study area

Age group (yrs)	Individuals examined			mf positive individual (%)			*mf geometric mean intensity			
	Female	Male	Total	Female	Male	Total	Female	Male	Total	mf positives only
1-14	242	252	494	2 (0.36)	3 (0.56)	5 (0.46)	1.05	1.05	1.05	127.85
15-24	114	137	251	0	5 (0.93)	5 (0.46)	0	1.18	1.05	86.40
25-34	57	44	101	0	0	0	0	0	0	0
35-44	42	30	72	1 (0.18)	0	1 (0.09)	1.10	0	1.06	51
45+	92	73	165	3 (0.55)	3 (0.56)	6 (0.55)	1.10	1.23	1.16	53.66
All	**547**	**536**	**1083**	**6 (1.10)**	**11 (2.05)**	**17 (1.57)**	**1.05**	**1.10**	**1.07**	**79.45**

*Antilog [Σ log (x + 1)/ n], where x is the number of mf per ml of blood in mf individuals and n is the number of people examined [9].

Molecular identification of *Wuchereria bancrofti* microfilariae in human blood
Microfilariae (mf) in human blood samples that were preserved in 3% acetic acid were also characterised after extracting the genomic DNA using the same kit described above. Infected blood samples were amplified and identified using the same procedure described in previous sections.

Ethical considerations
For the yearly MDA and mass screening for mf prevalence in the communities, oral informed consent was sought from all participants. Subsequently, written consent was obtained from each volunteer who slept under bed nets after the study purpose, procedures, entry and exit criteria were explained to them. All volunteers and the entire community members received that year's round of MDA immediately after the blood sample collection. The Institutional Review Board of Noguchi Memorial Institute for Medical Research approved the study.

Statistical analysis
Data were entered into Microsoft Access and analysed for the vector competency of *Anopheles* spp. in supporting the development of mf to the infective stage larvae. The same software was used to calculate the geometric mean intensity on mf in the human population. One-way analysis of variance (ANOVA) was used to test for the significance of age- and gender-specific variations between the human population and mf, with p value set at 0.05.

Results
Human microfilariae load in the communities after four rounds of MDA
The overall prevalence of mf in the study communities ($N = 1083$) was 1.6%; mf prevalence among males and females (2.05% and 1.10% respectively) was not significantly different ($p = 0.39$). The mf levels ranged from 0 to 59/100 μl blood with geometric mean intensity of 1.1 mf/ml of blood (Table 2). There was no significant variation in mf intensity and age-group ($p = 0.40$); likewise no significant difference between mf intensity and gender of participants ($p = 0.91$) (Table 2). Four out of the nine communities

namely Ayesuano, Dago, Hwida and Okyereko recorded positive cases, with Okyereko having the highest number of cases (Table 3). Among the positive cases, Okyereko recorded 155.6 mf/ml of blood whilst Dago recorded 15.3 mf/ml of blood.

Mosquito species composition and entomological indices
The 564 mosquitoes collected consisted of 350 (62.1%) *Anopheles*, 182 (32.3%) *Mansonia*, 28 (5%) *Aedes* and 4 (0.7%) *Culex* species, (Figure 1). The *Anopheles* species comprised of 310 (88.6%) *An. funestus,* 32 (9.1%) *An. gambiae* and 8 (2.3%) *An. Pharoensis,* (Figure 1). The hourly biting rates of *An. gambiae* and *An. funestus* were 6.4 and 62 bites/person/night respectively (Table 4). Of the mosquito species collected, 192 (34%) were engorged with blood-meals (Table 5). Whereas 6/350 (1.7%) of the *Anopheles* and 6/182 (3.3%) of the *Mansonia* species were found with the mf (L$_1$ stage) of *W. bancrofti*, there was no recovery of L$_3$ or L$_2$ stage larvae after 12 days of maintenance. While each of the infected *An. gambiae* had an average of one mf, each *An. funestus* had an average of eight mf when killed immediately after collection (Table 5). The mf load in the peripheral blood and biting rates of *Anopheles* mosquitoes peaked concurrently between 0.30 and 2.30 hours (Figure 2).

Table 3 Blood sampling results showing number of people infected with microfilaria of *Wuchereria bancrofti* in the year 2004 (four years of MDA)

Study communities	Number examined	Number positive (mf density/ml of blood)	Geometric mean intensity (mf/ml)
Amanful	61	0	0
Ayesuano	69	1 (4)	1.0
Dago	228	4 (1 – 15.3)	1.0
Fawomanyo	66	0	0
Hwida	88	2 (5 – 21)	1.0
Kyiren	161	0	0
Mampong	63	0	0
Obiri	70	0	0
Okyereko	277	10 (1 – 155.6)	1.2
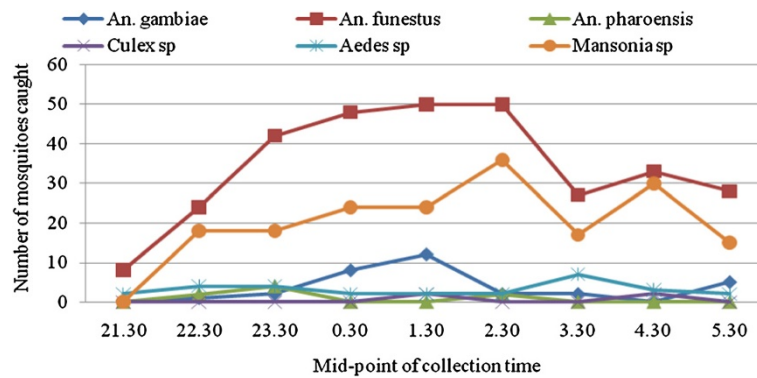
Figure 1 Hourly distribution of the mosquito species that were caught from the bed nets under which volunteers were sleeping.

PCR identification of *Anopheles* mosquitoes and *Wuchereria bancrofti*

Of the 32 *An. gambiae* s.l. collected, 30 were identified as *An. gambiae* s.s. as they showed the expected diagnostic band size of 390 base pairs. After restriction enzyme treatment with *Hha* I, M forms remained a single band of 390 bp since there was no digestion. S forms on the other hand resulted in two bands of 110 and 280 bp. Of the *An. gambiae* s.s digested, 21 (70%) were M forms with 9 (30%) S molecular forms. Among the 310 *An. funestus* s.l. collected, 286 were identified by PCR; of which 267 (86%) were *An. funestus* s.s. with diagnostic band sizes of 460 base pairs, and 19 (6%) were *An. Leesoni* with band sizes of 146 base pairs. The presence of *W. bancrofti* in 20 infected mosquitoes and 10 human blood samples were confirmed at 188 bp.

Discussion

An LF-endemic community is said to have low mf density when the density of circulating mf is less than 200 mf per ml of blood, an amount which cannot be detected in a significant number of instances when commonly used blood sampling techniques are employed [4]. Nonetheless, this depends on variables such as volume of blood examined, source of blood sampled (venous or capillary) and method of mf detection that is employed. In this study, 100 μl of finger-prick blood was used, which is an appreciable amount of blood compared to the popular technique for mf detection in routine public health practice of 20 μl finger-prick blood [4]. As such it could be inferred that the mean mf intensity of 1.07 and 79.45 mf per ml of capillary blood in the entire study communities and mf positive individuals respectively were really low in the studied area. This may be due to the 66.6% overall coverage rate in MDA with ivermectin and albendazole for 4 years leading to a reduction in mf densities among the inhabitants (Boakye DA, unpublished data). Evidence from Okyereko supports this view that MDA has been effective; in this study period, 155.6 mf per ml of blood were recorded among mf positive individuals, hitherto the commencement of MDA, as high as 819 mf/ml of blood were recorded among this group [12].

Various observations have been made regarding mf prevalence and intensities in study populations [9,11,12,22]. These could be attributed in part to the occupational activities of inhabitants of the study areas as well as the biting pattern of the local anopheline vectors, which are presently known to be the main vectors of LF in Ghana [10-12]. Studies on the relationship between mf density in blood meals and the percentage of *Anopheles* mosquitoes that

Table 4 Entomological indices of the various mosquito species that were caught during the study

Mosquito species	Entomological indices (%)					
	Biting rate[a]	Infection rate[b]	Infectivity rate[c]	Intensity of infection[d]	Survival rate[e]	Vector efficiency[f]
An. gambiae	6.4	0.13	0	0	0.50	0
An. funestus	62.0	0.03	0	0	0.47	0
An. pharoensis	1.6	0	0	0	1.0	0
Culex sp	0.8	0	0	0	0	0
Aedes sp	5.6	0	0	0	5.0	0
Mansonia sp	36.4	0.07	0	0	0.12	0

[a]Number of mosquitoes caught/ number of collectors x number of captures (bites/ person/ night); [b]Number of mosquitoes infected/ number of mosquitoes dissected; [c]L_3 in the head and proboscis/ number of surviving mosquitoes; [d]Number of mosquitoes with L_3 in the head and proboscis/ number of mosquitoes with L_3; [e]Number of surviving mosquitoes at the end of study/ number of engorged mosquitoes. [f]Number of L_3 x 100/ number of mf ingested among dissected mosquitoes [19-21].

Table 5 Number of mosquitoes caught and examined before and after maintenance in the laboratory

Mosquito species	No: of mosquitoes		No: of mosquitoes examined immediately after collection		No: of mosquitoes examined from days 1-8 of maintenance		No: of mosquitoes examined from days 9-13 of maintenance	
	Caught	Engorged with blood	Dissected	Infected (no: mf)	Dissected	Infected (no: mf)	Dissected	Infected
An. gambiae	32	12	16	2 (2)	10	2 (3)	6	0
An. funestus	310	98	155	4 (33)	109	4 (4)	46	0
An. pharoensis	8	2	4	0 (0)	2	0	2	0
Culex sp.	4	2	2	0 (0)	2	0	0	0
Aedes sp.	28	2	14	0 (0)	4	0	10	0
Mansonia sp.	182	76	90	6 (12)	82	7 (7)	9	0
Total	**564**	**192**	**281**	**12 (47)**	**209**	**13 (14)**	**73**	**0**

All mf found in mosquitoes were of L_1 stage, neither L_2 nor L_3 stages were found. All mosquitoes caught were dissected latest by end of the maintenance (13 days).

ingest mf have not provided consistent results [3,9,22-25]. Southgate and Bryan [26] showed that although many of the mf ingested by *Anopheles* vectors are damaged by the mosquito's foregut armature, the proportion of mf destroyed does not depend on the number of mf ingested and varies between members of the *An. gambiae* complex and *An. funestus*. It is therefore not proper to extend findings from a given area to the other even for the same species. Additionally, other anatomical structures and immune factors other than the foregut armature could be modulating mf density following ingestion. Further studies are thus required to provide more understanding into the vector-parasite relationships.

Indeed the significance of distinctly different host-parasite relationships lies in the importance of low-density mf in sustaining transmission in various endemic areas with different genera of mosquito vectors [4]. As hypothesised, the theory of "limitation" allows transmission to occur and build-up when most infected human hosts have low mf densities, whereas situations of well marked "facilitation" will give rise to transmission thresholds below

which transmission will ultimately cease leading to parasite elimination from the human population. However, such predictions of parasite extinction or parasite resurgence can only be made with confidence when characteristics of the local vector-mf relationship are well understood [7].

As part of our study, we described the circadian pattern of mf periodicity in southern Ghana. Based on hourly examination of twelve volunteers for nine hours, we observed that mf concentration in peripheral blood followed a wave-like concentration peaking around 01.00 hours, which was similar to other findings [12,27,28]. This interesting behavioural pattern of mf is said to be the parasite's response to oxygen tension, which is high in peripheral circulation at night due to the low human activity at this time of the day [29,30].

In their study, Dzodzomenyo M. et al. [12] observed *An. funestus* to be the most abundant mosquito species in the early dry season while *An. gambiae* was predominant in the wet season. Our study was conducted in March, which is the peak of the dry season in Ghana and thus may contribute to the low number of *An. gambiae* that were
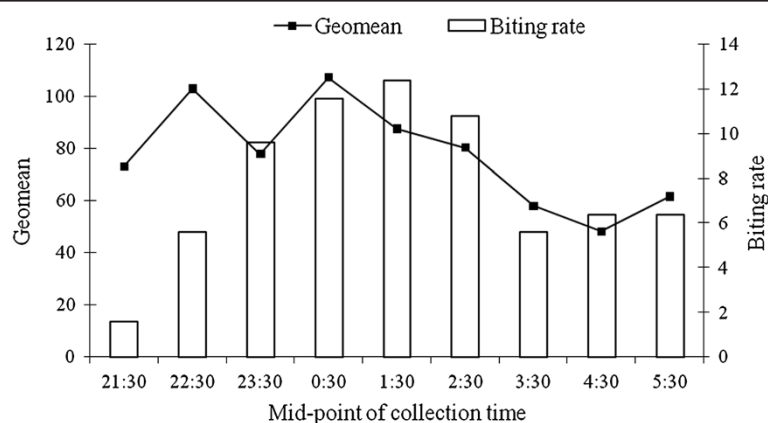
Figure 2 Biting rate of *Anopheles* species and the geometric mean intensity (Geomean) of mf that were observed during the night of sample collection.

captured. Studies show that the M and S forms of *Anopheles gambiae* s.s. do occur in sympatry in southern Ghana [31]. Our study revealed that most of the *Anopheles gambiae* s.s. were M form, which has a remarkable ecological flexibility and is known to prevail in inundated areas where dry season breeding opportunities exist [10]. Further studies could look at the role of these molecular forms of *Anopheles gambiae* s.s. in transmission of *W. bancrofti* following MDA.

Conclusion

After 4 rounds of mass drug administration, parasitaemia was brought to a low level in the study communities. Low levels of circulating microfilariae in the inhabitants might have contributed to the no recovery of infective stage larvae of *W. bancrofti* in *An. funestus* s.l. as well as M and S forms of *An. gambiae*. Although the mosquito numbers were low, a further study is recommended to ascertain this observation.

Authors' contributions
All authors contributed significantly to this study. DAB and MDW conceived the idea and design of the study. BKB, FAA and JO carried out the field and laboratory studies. BKB prepared the manuscript, while all authors read and approved the final manuscript.

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