RESEARCH ARTICLE

REVISED Multiple insecticide resistance and *Plasmodium* infection in the principal malaria vectors *Anopheles funestus* and *Anopheles gambiae* in a forested locality close to the Yaoundé airport, Cameroon [version 2; peer review: 2 approved]

Previously titled: Elevated *Plasmodium* sporozoite infection and multiple insecticide resistance in the principal malaria vectors *Anopheles funestus* and *Anopheles gambiae* in a forested locality close to the Yaoundé airport, Cameroon

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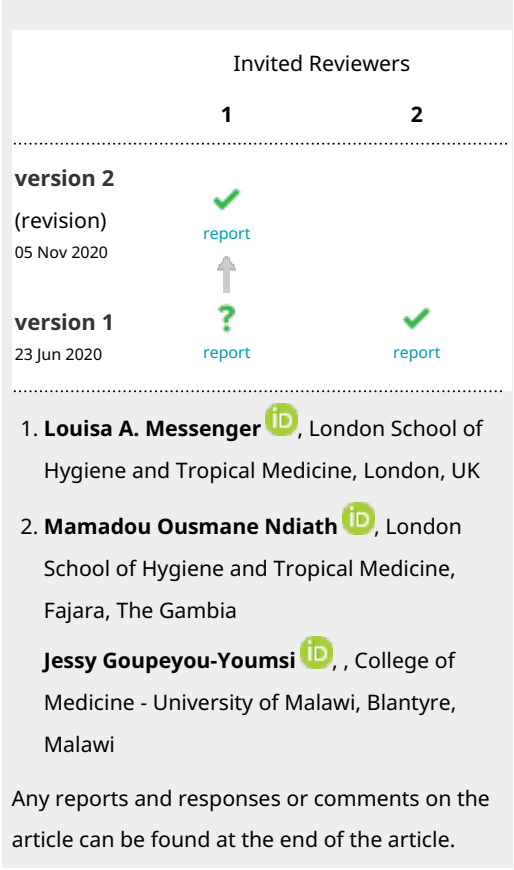
Abstract

Background: Reducing the burden of malaria requires better understanding of vector populations, particularly in forested regions where the incidence remains elevated. Here, we characterized malaria vectors in a locality near the Yaoundé international airport, Cameroon, including species composition, abundance, *Plasmodium* infection rate, insecticide resistance profiles and underlying resistance mechanisms.
Methods: Blood-fed adult mosquitoes resting indoors were aspirated from houses in April 2019 at Elende, a locality situated 2 km from the Yaoundé-Nsimalen airport. Female mosquitoes were forced to lay eggs to generate F_1 adults. Bioassays were performed to assess resistance profile to the four insecticides classes. The threshold of insecticide susceptibility was defined above 98% mortality rate and mortality rates below 90% were indicative of confirmed insecticide resistance. Furthermore, the molecular basis of resistance and *Plasmodium* infection rates were investigated.
Results: *Anopheles funestus* s.s. was the most abundant species in Elende (85%) followed by *Anopheles gambiae* s.s. (15%) with both having similar sporozoite rate. Both species exhibited high levels of resistance to the pyrethroids, permethrin and deltamethrin (<40% mortality). *An. gambiae* s.s. was resistant to DDT (9.9% mortality) and bendiocarb (54% mortality) while susceptible to organophosphate. *An. funestus* s.s. was resistant to dieldrin (1% mortality), DDT (86% mortality) but susceptible to carbamates and organophosphates. The L119F-GSTe2 resistance allele (8%) and G119S *ace*-1 resistance allele (15%) were detected in *An. funestus* s.s. and *An. gambiae* s.s., respectively. Furthermore, the high pyrethroid/DDT resistances in *An. gambiae* corresponded with an increase frequency of 1014F *kdr* allele (95%). Transcriptional profiling of candidate cytochrome P450 genes reveals the over-expression of *CYP6P5*, *CYP6P9a* and *CYP6P9b.*
Conclusion: The resistance to multiple insecticide classes observed in these vector populations alongside the significant *Plasmodium* sporozoite rate highlights the challenges that vector control programs encounter in sustaining the regular benefits of contemporary insecticide-based control interventions in forested areas.

Keywords
Malaria, Anopheles funestus, Anopheles gambiae, Plasmodium infection, Insecticide resistance, Vector control, Cameroon



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REVISED **Amendments from Version 1**

In the present version of the manuscript we took into account the remarks made by the reviewers to improve on the scientific quality of the paper. Regarding the major comments, we acknowledged the small sample size of *An. gambiae* in the discussion section in line with the results obtained. We also included the number of mosquitoes tested per insecticide treatment for both the insecticide susceptibility and PBO assays. Moreover, we also submitted the ITS2 sequences to GenBank.

Any further responses from the reviewers can be found at the end of the article

Abbreviations

DDT: dichlorodiethyltrichloroethane; DNA: deoxyribonucleic acid; dNTPs: deoxyribonucleoside triphosphates; GSTe2: glutathione S-transferase epsilon 2; IRS: indoor residual spraying; kdr: knockdown resistance mutation; LLIN: long-lasting insecticidal net; NMCP: National Malaria Control Programme; PBO: piperonyl butoxide; PCR: polymerase chain reaction; *s.l.*: *sensu lato*; s.s.: *sensu stricto;* WHO: World Health Organization.

Introduction

Malaria is the major vector-borne disease globally and a leading public health problem[1]. In 2018, there were roughly 228 million cases of the disease and about 405,000 malaria-related deaths. Approximately 67% of deaths recorded were children aged below five years[1]. Although, a shift in focus from malaria control to elimination was declared by the WHO in 2012, it was observed that between 2015 and 2018; no considerable progress was achieved in decreasing global malaria cases. Rather, there was a reported increase in malaria victims in 2018 compared with the previous years in ten African countries scoring the highest burden of the disease[1].

To this effect, the WHO Global Technical Strategy for Malaria (2016–2030) outlines a pathway for malaria control and elimination and designates a target for a 90% reduction in global malaria mortality rates by 2030 relative to a 2015 baseline[2]. In this vein, the recent certification of Algeria and Argentina as malaria-free countries by the World Health Organization (WHO) has been a historic achievement for universal health coverage, and serving as a model in demonstrating the feasibility of malaria elimination in the Afro-tropical region[1]. This success was in part largely attributed to a coordinated system of vector control interventions such as long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) among others, including prompt diagnosis, effective treatment and efficient surveillance response system[3]. Despite this, the efficacy of these insecticide based-vector control tools is compromised by the growing problem of insecticide resistance widely exhibited by *Anopheles* vectors across the African continent[4].

In Cameroon, malaria is endemic, with the entire population considered to be at risk. In 2018, the country accounted for 3% (6,840,000) of malaria cases and recorded about 3,000 deaths within the WHO African region[1]. In order to reduce the malaria burden, the Cameroonian government supports the National Malaria Control Program (NMCP) and other partners who have established a strategic plan to achieve the goal of ensuring equal access to quality and affordable tools necessary for sustaining malaria control and elimination. The malaria interventions include mass distribution of LLINs, prompt and effective diagnosis, artemisinin-based combination therapy (ACTs), seasonal malaria chemoprevention (SMC) and intermittent preventive therapy (IPT) in pregnant women through administration of sulfadoxine-pyrimethamine[5]. Moreover, to further strengthen the current vector control intervention, an IRS campaign is to be launched in Cameroon for the first time by the United States President Malaria Initiative (PMI) project. Unfortunately, the wide spread nature of insecticide resistance in malaria vectors to common insecticides used to impregnate nets and spray walls in Cameroon[5–10] threatens the success of this strategy.

In Cameroon, malaria is mainly transmitted by *An. gambiae s.s, An. coluzzii, An. arabiensis, An. funestus* s.s., *An. nili* and *An. moucheti vectors*[5] which have different geographical distributions across the country. However, despite such an important epidemiological role played by these vectors, data on the pattern of malaria transmission across different ecological settings in Cameroon including urban, peri-urban and rural remains insufficient[11]. This represents a major challenge for the realization of effective universal coverage of LLINs and universal access to anti-malaria drugs and treatment since malaria control requires a good understanding of the transmission dynamics. Currently, the absence of sufficient information on vector bionomics and disease transmission in peri-urban areas could constitute a potentially steady malaria pool. This may serve as a bridge between rural and urban regions especially if high or residual transmission are maintained in such areas[12] thus posing a massive challenge for malaria control.

In order to facilitate and reinforce the National Malaria Control Program in their efforts to implementing sustainable and efficacious vector control interventions, this study investigated the entomological component of malaria transmission in a peri-urban setting within the forested region of Cameroon. This included the characterization of the endophilic mosquito species composition, investigation of the insecticide resistance profiles of *An. funestus* s.s. and *An. gambiae* s.s. populations collected in Elende in 2019, a forested peri-urban area located 2 km away from the Nsimalen International Airport of Yaoundé, the capital city of Cameroon.

Methods

Study area and mosquito collection

Blood fed female mosquitoes were collected indoor in April 2019 from Elende (3°41’57.27’’N, 11°33’28.46’’E) (Figure 1), district of Nkolmefou I, a peri-urban locality close to Yaoundé; the capital city of Cameroon. This area is about 2 km away from the Nsimalen International Airport and close to the Mefou River. This locality is categorized by an equatorial Guinean climate, represented by two rainy seasons (August-October and March-May) and two dry seasons (November-February and June–July). The yearly average rainfall is 1800 mm

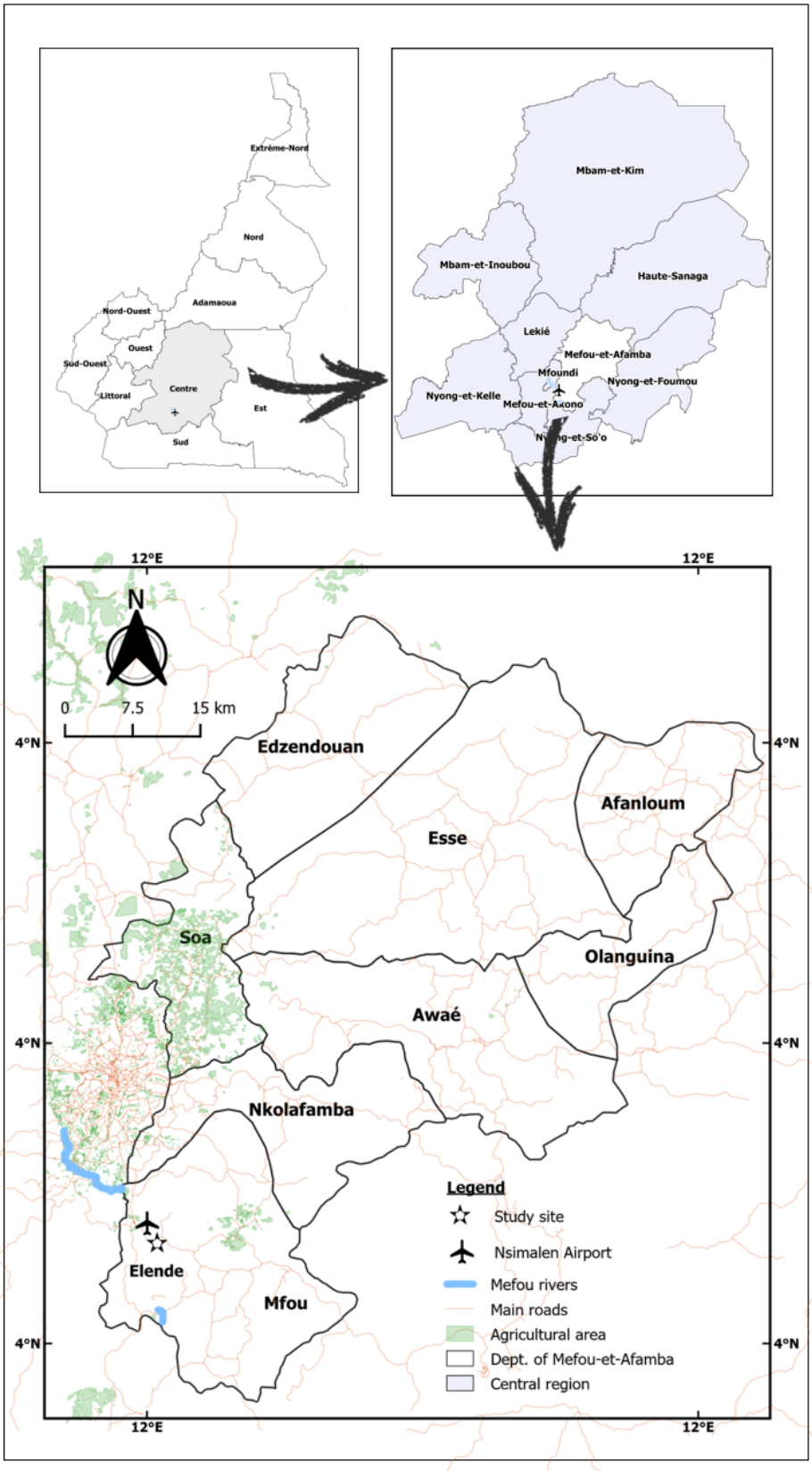

Figure 1. Map of Elende study area; showing its close proximity to the Nsimalen- International Airport of Yaoundé, capital city of Cameroon.

while mean annual temperatures range between 19–28°C, and the mean humidity varies between 65–80%. The vegetation around the village is predominantly made up of an equatorial forest which is being degraded for farming activities and infrastructure. Road construction activities and deforestation are ongoing in this locality, an environmental modification system which creates temporal and permanent breeding sites for malaria vectors such as *An. gambiae* s.s. Moreover, the village is proximal to marshy lands and streams joining major rivers as these persistent water masses are ideal conditions, particularly favoring vector multiplication of *An. funestus* s.s. mosquitoes. Subsistence farming including cassava cultivation and vegetable cropping (particularly tomatoes, pepper, lettuce and watermelon) are the main human activities in this locality. The yield from this farming practices are greatly enhanced by the intensive use of pesticides. Also, household animals such as cattle, goats and sheep are present on a minor scale. Furthermore, several fish ponds bordered with vegetation exist in this village. This might encourage the development and growth of immature stages of the species within the *An. funestus* group. In this locality, pyrethroids containing LLINs (PermaNet 2.0) is the main prevention method with a coverage of around 60%[5] as the area is endemic for malaria.

Following verbal approval from the chief and household heads; blood-fed indoor resting adult mosquitoes (F0s) were collected between 06.00am and 11.00am from 15–20 randomly selected houses to avoid sampling individuals from single female egg batches and to obtain a representative population-level data for this area. The Prokopack electrical aspirator (John W Hock Co, Gainesville, FL, USA) was used for mosquito collection, after which they were kept in a humid cage and later transported to the insectarium of the Centre for Research in Infectious Diseases (CRID), Yaoundé. Field collected females were placed in a cage to rest for 1 hour prior to morphological identification[13]. Each live mosquito sample was aspirated from the cage in to a hemolysis tube and observed microscopically for distinct morphological differences of wings, mouthparts and size based on the Afro-tropical anopheline key[13]. Specimens of the *An. funestus* group and *An. gambiae* complex were then placed in two separate small-sized labeled cages and left for 4–5 days feeding on 10% sugar soaked in cotton wool for them to become fully gravid. A forced-egg laying method as previously described[14,15] was utilized for individual oviposition of females. After oviposition, all the carcasses of the F_0 were kept in separate 1.5-ml tubes containing silica gel and stored at -20°C prior to molecular analysis.

Molecular species identification

The Livak method was used to extract genomic DNA from the head/thorax of each individually oviposited and non-oviposited field-caught female (F_0s)[16]. DNA extracts were quantified using NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, USA). A SINE-200 PCR[17] and cocktail PCR[18] were performed to identify the different species within the *An. gambiae* s.l. complex and *An. funestus* s.l. group, respectively. The ribosomal DNA internal transcribed spacer region 2 (ITS2) was amplified to identify the undetermined species using the protocol of Hackett *et al.*[19]. The PCR amplicon of 840 bp was purified with Exonuclease I (Exo I) and Shrimp Alkaline Phosphate method (Exo-SAP) based on the New England Biolabs procedure (NEB, MA, USA) and directly sequenced commercially.

Plasmodium infection rates

Plasmodium infection was assessed in 150 *An. funestus* s.s. and 39 *An. gambiae* s.s. F0 females. Genomic DNA was extracted from the head and thorax of each specimen, and infection with *P. falciparum* or OVM+ (*P. ovale, P. vivax* and *P. malariae*) was detected using the TaqMan assay as described previously with slight modifications (1 cycle at 95°C/10mins and 40 cycles at 92°C/15s and 60°C/1min)[20]. Sequentially, nested PCR was conducted on all the positive samples to confirm the TaqMan assay results and to specifically differentiate between the OVM species obtained[21].

WHO insecticide susceptibility bioassays

Various insecticides employed in control of malaria vectors were tested in bioassays to assess the resistance profile of the *An. funestus* s.s. and *An. gambiae* s.s. mosquito populations, according to the WHO protocol[22]. Brand new insecticide-impregnated papers were supplied by WHO reference center (Vector Control Research Unit, University Sains Malaysia, Penang, Malaysia). Two- to five-day-old, unfed F_1 female *An. funestus* s.s. were exposed for 1 h to discriminating concentrations of the following insecticides: pyrethroids [the class I pyrethroid permethrin (0.75%); n=80, and the class II pyrethroid deltamethrin (0.05%); n=78]; the organochlorines [DDT (4%); n=80 and dieldrin (4%); n=95]; the carbamates [bendiocarb (0.1%); n=79 and propoxur (0.1%); n=87]; and the organophosphate malathion (5%) (n=99). The remaining population constituted the control group (n=40). Similarly, *An. gambiae* s.s. female mosquitoes were exposed to all the insecticides [permethrin (n=86), deltamethrin (n=89), DDT (n=90), bendiocarb (n=87), malathion (n=84)] except dieldrin and propoxur; instead, fenithrothion (5%) (n=84) and pirimiphos-methyl (1.25%) (n=61) were added.

Furthermore, in order to establish the resistance intensity to pyrethroid insecticides, the *An. gambiae* s.s. F_1 generations from Elende were tested on permethrin (n=171) and deltamethrin (n=167) concentrations of 5x and 10x for 60 mins. Mortality rates were recorded 24 h post exposure. The dead mosquitoes were kept in 1.5-ml tubes containing silica gel while the survivors were placed in tubes containing RNALater and stored at -80°C for molecular analyses.

A set of 20-25 mosquitoes exposed to untreated papers were used as control for each test. The experiment was carried out at ambient temperatures of 25°C ± 2°C and 80% ± 10% relative humidity. A mortality rate >98% of the mosquito populations was considered susceptible to the insecticide, meanwhile suspected resistance was considered at mortality between 90–98%, and resistant where mortality was found to be <90%.

Piperonyl butoxide (PBO) synergist assay

Only females of *An. funestus* s.s. were used for this assay since *An. gambiae* s.s. was inadequate in the study area at the period of collection. In order to determine the possible implication of cytochrome P450s in the observed phenotypic resistance to pyrethroids, two to five days old F_1 female *An. funestus* s.s. were initially exposed to 4% PBO for 1 h proceeded by immediate second exposure to permethrin (0.75%) or deltamethrin (0.05%) for another 1 h exposure. The mortality was determined at 24 h post exposure and compared with mortality achieved for mosquitoes subjected to the pyrethroids only. Differences in mortality among the various groups were analyzed and recorded[22].

Assessment of bed net efficacy using cone assay

Due to the low abundance of *Anopheles gambiae* s.s., in this locality, we examined the efficacy of bed nets approved by WHO against the Elende *An. funestus* s.s. population. This was done to evaluate the impact of resistance on insecticide targeted interventions against *Anopheles* vectors in this village. Cone bioassays were performed according to the WHO procedure[23] using four standard types of LLINs (Olyset Plus, Olyset Net, PermaNet 2.0 and PermaNet 3.0 (side and roof)). Brand new PermaNet and OlysetNet nets manufactured in 2018 were supplied by the companies, Vestergaard (Lausanne, Switzerland) and Sumitomo Chemical Plc (London, UK), respectively. Four replicates of ten unfed mosquitoes were introduced into each plastic cone attached on pieces of fresh, unused bed nets of dimension 30 cm × 30 cm: Olyset®Net (containing 2% permethrin), OlysetPlus®Net (containing 2% permethrin combined with 1% of the synergist PBO), PermaNet®2.0 (containing 1.4–1.8 g/kg ± 25% deltamethrin), and PermaNet®3.0 [both the side panel (containing 2.1–2.8 g/ kg ± 25% deltamethrin) and the roof (containing 4.0 g/kg ± 25% deltamethrin, combined with 25 g/kg ± 25% of PBO)]. In a similar way, four replicates of ten mosquitoes each were included in each batch of the LLIN cone test and exposed to an untreated net to serve as negative control. For each test, 3 min was the exposure time. After exposure, the mosquitoes were gently and immediately removed from the cones using a mouth aspirator, transferred into paper cups and fed with 10% sucrose soaked in cotton wool. The number of mosquitoes knocked-down was recorded after 1 h while mortality was calculated after 24 h of observation. The experiment was carried out at ambient temperature of 25°C ± 2°C and 80% ± 10% relative humidity.

Genotyping of L119F-GSTe2, *CYP6P9a*, A296S-RDL, resistance marker in *Anopheles funestus s.s*

An allele-specific PCR was used to genotype and determine the frequency of the L119F-GSTe2 mutation in *An. funestus* s.s. F_0 mosquito population of Elende as previously described[24,25]. This was to investigate the role of glutathione S-transferases in DDT resistance. The presence of *CYP6P9a* resistance allele associated with resistance to pyrethroids was genotyped by a PCR-RFLP assay[26] while the A296S-RDL mutation known to be linked with dieldrin resistance was also genotyped by TaqMan assay[27].

Genotyping of L1014F, L1014S *kdr* and G119S *ace-1* resistance marker in *Anopheles gambiae* s.s.

The L1014F-kdr and L1014S-kdr mutations involved in pyrethroid and DDT resistance in *An. gambiae* s.s. were genotyped in F_0 Elende mosquitoes using the TaqMan assay[28]. In addition, the G119S *ace-1* responsible for carbamate and organophosphate resistance in *An. gambiae* s.s. was also genotyped in Elende mosquitoes using a TaqMan assay as previously described[29].

Transcription profiling of candidate resistance associated genes

A quantitative reverse transcriptase PCR (qRT-PCR) was done to investigate the prominent role of some previously reported Cytochrome P450 detoxification genes (*CYP325A*, *CYP6P5*, *CYP6P9a* and *CYP6P9b*) in *An. funestus* s.s. associated to the phenotypic resistance recorded during bioassay[30–32]. Using a triplicate of 10 F_1 females each that recovered after 1 h exposure to permethrin from Elende and 3 batches of unexposed 10 F_1 females that were used as control samples; total RNA extraction, cDNA synthesis and qRT-PCR reactions were performed as earlier reported[30]. Fold change and expression of each gene in resistant (R) and control (C) samples were computed according to 2-ΔΔCq method[33] following standardization with housekeeping gene: ribosomal protein S7 (RSP7) (AFUN007153-RA) and the Actin 5C (AFUN006819) genes.

An earlier version of this article can be found on Research Square (DOI: https://doi.org/10.21203/rs.2.23277/v1).

Results

Species identification

A total of 269 adult resting female *Anopheles* mosquitoes were collected indoors at Elende over a two-day period. Out of the total sample collected; 230 (85.50%) were members of *An. funestus* group while the remaining 39 (14.49%) species belonged to the *An. gambiae* complex.

From the 120 *An. funestus* s.l. mosquitoes that were chosen randomly and identified using cocktail PCR, *An. funestus* s.s. was dominant [98.34% (118/120)] with *An. rivolurum* [0.83% (01/120)] and *An. vaneedeni* [0.83% (01/120)] also detected. Similarly, from the 39 *An. gambiae s.l.* mosquitoes collected and analyzed using SINE PCR, all were *An. gambiae* s.s. Furthermore, the results of ITS-2 sequencing confirmed the undetermined mosquitoes from cocktail PCR to be *An. funestus* s.s [GenBank accession numbers: MT991011 to MT991016]. Out of the 230 and 39 mosquitoes morphologically identified, 196 (85.22%) *An. funestus* s.s. and 20 (51.28%) *An. gambiae* s.s. laid eggs respectively by the forced egg laying technique (see *Underlying data* for raw PCR values[34]).

Plasmodium infection rate

The analysis of the head and thorax revealed 09/150 (6%, CI: 4.15-8.57) infected *An. funestus* s.s. mosquitoes, which included 7 (4.67%, CI: 2.35-7.16) *P. falciparum* and 2 (1.33%, CI: 0.66-2.97) mosquitoes infected with either *P. ovale*, *P. vivax*

or *P. malariae* (OVM). The *Plasmodium* infected mosquitoes revealed by TaqMan were further tested with nested PCR. Results of nested PCR reported 5 *P. falciparum* positive samples and 2 samples infected with *P. malariae*.

Out of 39 collected *An. gambiae* s.s., 5.13% [(02/39) (CI: 2.57-7.13)] were infected with *P. falciparum* and none for *P. OVM* or mixed infections. Nested PCR confirmed 1 positive *P. falciparum* sample out of the 2 infected samples detected by TaqMan assay (see *Underlying data* for raw values[34]).

Insecticide susceptibility assays

A sum of 638 F_1 female *An. funestus* s.s. mosquitoes were tested to determine the resistance profile to seven insecticides (Figure 2). *An. funestus* s.s. was resistant to class I and class II pyrethroids. Mortality to permethrin (type I pyrethroid) was 37.00±6.59% while for deltamethrin (type II pyrethroid), mortality was 29.80±0.86% (Figure 2a). Regarding the organochlorine insecticides, moderate resistance to DDT was recorded with a mortality rate of 86.25±8.98% (Figure 2a). However, extremely high resistance was noticed for dieldrin with a recorded mortality value of 1.04±1.04% (Figure 2a). For the carbamates, full susceptibility was observed for propoxur with a mortality rate of 100% whereas a near susceptibility was noted for bendiocarb with a mortality value of 97.37±2.63% (Figure 2a). A full susceptibility was obtained for the organophosphate, malathion with 100% mortality (Figure 2a).

A total of 979 female F1 *An. gambiae* s.s. progeny was tested to evaluate the resistance profile to seven insecticides as well as the resistance intensity to the pyrethroids. The *An. gambiae* s.s. population was highly resistant to class I and class II pyrethroids. Mortality to permethrin was 5.00±0.06% and 11.32±1.40% for deltamethrin (Figure 2b). Due to the high resistance observed for the diagnostic concentration of pyrethroids, an increased concentration of permethrin (5x and 10x) and deltamethrin (5x and 10x) was used to further determine the extent of resistance intensity on the F_1 *An. gambiae* s.s. population. High intensity resistance to 5x and 10x permethrin was observed with a recorded mortality rate of 31.74% and 44.30% respectively which according to the WHO guidelines[22] indicates a high intensity of resistance in Elende. In a

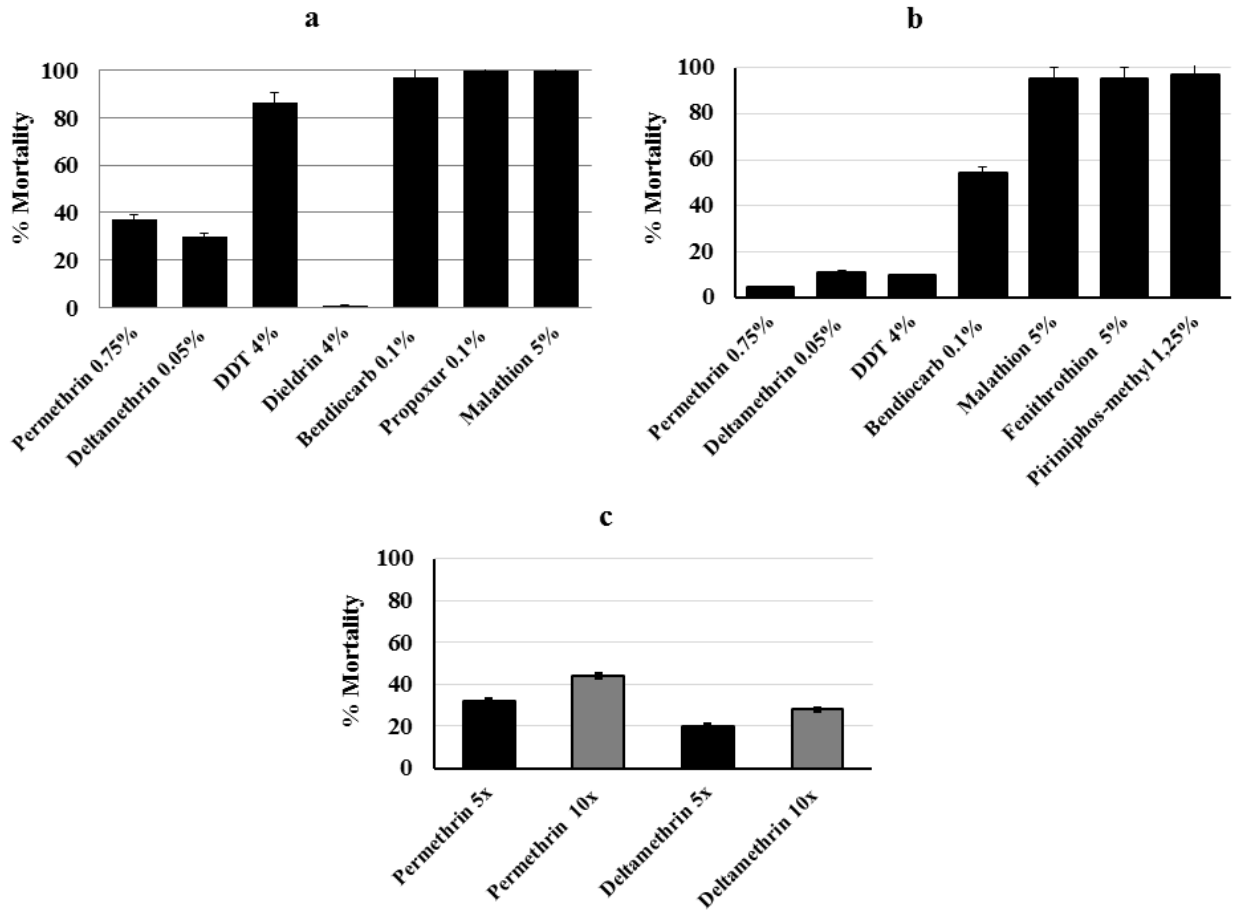

Figure 2. Results of WHO insecticides susceptibility test. (**a**) Susceptibility profile of female *An. funestus* s.s. (F1 progeny; N=638) from Elende following exposure to various public health insecticides. (**b**) Susceptibility profile of Elende female *An. gambaie* s.s. (F1 progeny; N=621). (**c**) Resistance intensity of *An. gambiae* s.s. (F1; N=358) on exposure to 5x and 10x permethrin and deltamethrin each; N is the total number of mosquitoes tested. Error bars represent standard error of the mean.

similar manner, mortality rate upon exposure to 5x and 10x deltamethrin was observed at 19.80% and 29.00%, respectively indicating a high intensity resistant population (Figure 2b). Regarding the organochloride insecticide DDT, an elevated resistance pattern was observed with a mortality rate of 9.98±1.03% (Figure 2b). Resistance to the carbamate insecticide, bendiocarb was recorded with a mortality value of 54.56±1.05% (Figure 2b). Moreover, for the organophosphate insecticides, mortality rates of 95.24±1.94%, 95.23±0.09%, and 96.75±1.63% were observed for malathion, fenithrothion and pirimiphos-methyl respectively (Figure 2b) suggesting possible resistance that necessitates further confirmation (see *Underlying data*[34]).

PBO synergist assays with *An. funestus* s.s.

A total of 489 female F1 progeny was employed for the PBO synergist assay. Initial exposure of 169 F_1 *An. funestus* s.s. mosquitoes to PBO led to return of susceptibility to both class I and II pyrethroids with full mortality recorded after PBO + pyrethroid exposure from 51.50±7.19% to 100% for permethrin and from 19.22±10.76% to 100% mortality for deltamethrin (Figure 3a) (see *Underlying data*[34]). Briefly, the number of mosquitoes per treatment includes: permethrin (n=85), PBO + permethrin (n=80), deltamethrin (n=96), PBO + deltamethrin (n=89), PBO only (n=74) and control (n=65).

Assessment of bed net efficacy on *An. funestus* s.s. population by cone assays

A total of 230 F1 female mosquitoes were used for the cone assay. Out of the 192 F1 *An. funestus* s.s. used to assess the efficacy of conventional bed nets, a reduced efficacy was seen for both pyrethroid-only impregnated nets with low mortality rate observed for Olyset net (24.97±6.45%) and PermaNet 2.0 (7.27±4.75%) 24 h after mosquito exposure (Figure 3b). Conversely, nets impregnated with PBO exhibited a significantly greater efficacy with full susceptibility for both PermaNet 3.0 top (100% mortality) and Olyset plus (100% mortality). However, a lower efficacy was recorded for the side part of PermaNet 3.0 (19.17 ± 5.46% mortality). The susceptible strain for *An. funestus* FANG, used as control demonstrated full mortality to all nets (Figure 3b) (see *Underlying data*[34]).

Genotyping of L119F GSTe2, Cyp6P9a and A296S-RDL resistance markers in *An. funestus* s.s.

A subset of 50 F_0 females collected from the field was used for genotyping L119F-GSTe2 molecular marker (Figure 4a). Out of this cohort, 04 mosquito samples were homozygous resistant (RR) (8%), 40 were heterozygous (RS) (80%) and 6 were homozygous susceptible (SS) (12%). Overall, the frequency of the 119F resistant allele (R) was 48% and 52% for the L119 susceptible allele (S).

The PCR-RFLP genotyping of *CYP6P9a* revealed that all the mosquitoes were homozygous susceptible with a band size of about 500 bp (Figure 4a) indicating that this mutation is absent in *An. funestus* s.s. population of Elende.

Also, the 50 samples genotyped for A296S marker were all homozygous resistant RR (100%), revealing that the mutation is fixed in this population (Figure 4a) in line with the high dieldrin resistance observed (see *Underlying data*[34]).

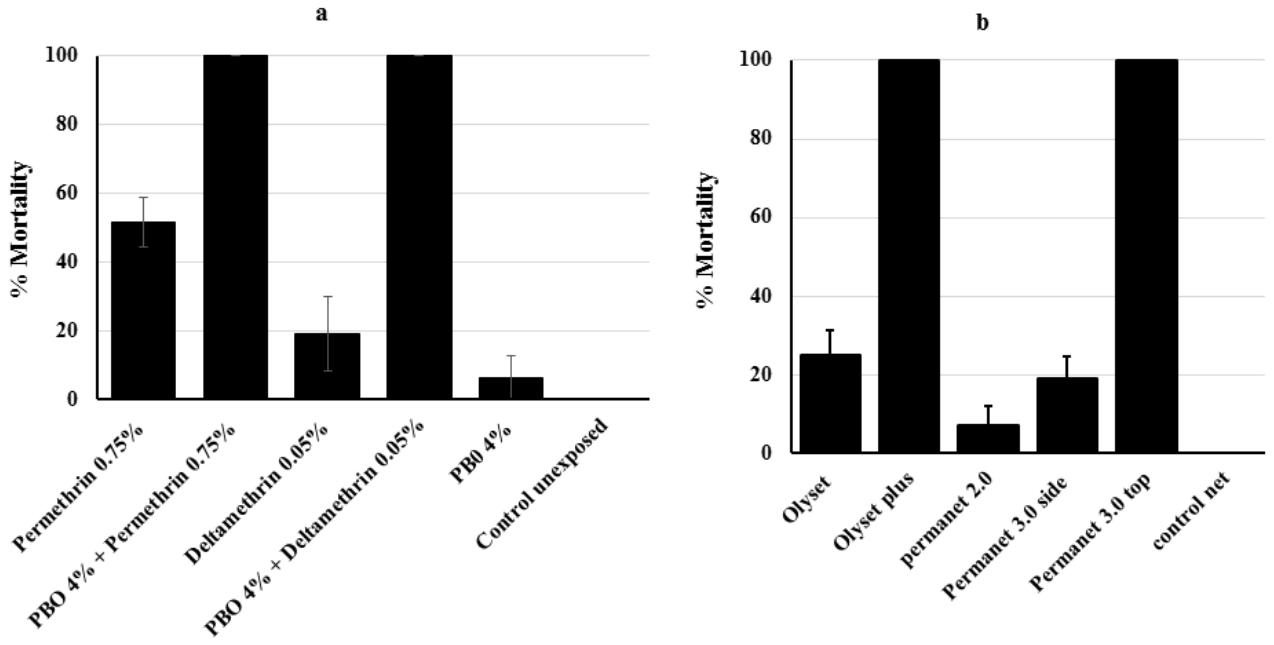

Figure 3. Susceptibility profile of *An. funestus* s.s. to synergist and cone assays from Elende. (**a**) Activities of PBO synergist assay on *An. funestus* s.s. (F1 population; N=489). (**b**) Recorded mortalities following 3-min exposure by cone assay of *An. funestus* s.s. (F1 generation; N=230) from Elende to Olyset, Olyset Plus, PermaNet 2.0, PermaNet 3.0 (side) and PermaNet 3.0 (roof); N is the total number of mosquitoes tested. Data are shown as mean±SEM.

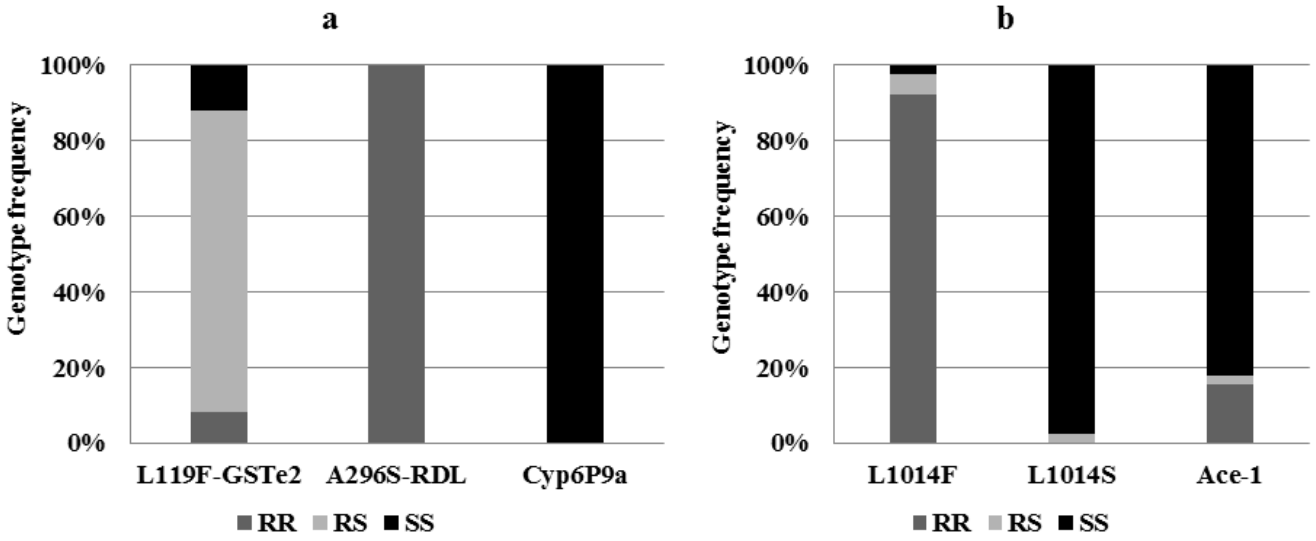

Figure 4. Temporal distribution of resistance markers in Elende. (**a**) *An. funestus s.s*: Frequency of the L119F-GSTe2 conferring DDT resistance; A296S-RDL mutation conferring dieldrin resistance; Cyp6P9a related with pyrethroid resistance. (**b**) *An. gambiae s.s*: Frequency of the L1014F conferring pyrethroids and DDT *kdr*W resistance; L1014S related with pyrethroids and DDT *kdr*E resistance; G119S *ace-1* conferring carbamate and organophosphate resistance. RR, homozygote for resistant allele; RS, heterozygote; SS, homozygote for susceptible allele.

Genotyping of L1014F, L1014S *kdr* and G119S ace 1 markers in *An. gambiae* s.s.

Out of 39 samples genotyped for L1014F *kdr* resistance marker, 36 were homozygous resistant RR (92.30%), 02 were heterozygous RS (5.13%) and 01 homozygous susceptible SS (2.56%) with a 1014F resistant allele frequency of 94.86% (Figure 4b). Likewise, out of the 39 samples genotyped for the L1014S marker, 01 was RS (2.56%) and 38 were SS (97.43%) (Figure 4b). Thus, a very low frequency of 1.28% was observed for the1014S resistant allele.

Similarly, out of 39 samples genotyped for the G119S resistance marker, 06 were homozygous resistant RR (15.38%), 01 was heterozygous resistant RS (2.56%) and 32 were found to be homozygous susceptible SS (82.05%) (Figure 4b). Overall, the frequency of the 119S resistant allele was 16.66% (see *Underlying data*[34]).

Transcriptional profiling of candidate genes

qRT-PCR was done to examine the role of some previously reported Cytochrome P450 metabolic genes in *An. funestus* s.s. linked to the resistance observed during bioassay (see *Underlying data*[34]). The qRT-PCR results reveal that *CYP6P5*, *CYP6P9a* and *CYP6P9b* genes known to be involved in pyrethroid resistance are significantly up-regulated in *An. funestus* s.s. population from Elende as compared to the susceptible laboratory strain FANG (Figure 5). Both *CYP6P9a* and *CYP6P9b* exhibited a 6-fold change in Elende resistant mosquito when compared to FANG ($P<0.05$) whereas *CYP6P5* displayed a 2.20-fold change difference in expression ($P<0.05$) between the wild mosquitoes and susceptible strain. On the other hand, when comparing permethrin exposed to the unexposed (control) mosquito and FANG strain, *CYP325A* was not significantly expressed.

Discussion

Rapid scaling up of vector control interventions is ongoing in Cameroon, where malaria is highly endemic. As such, characterization of local vectors alongside investigation of their resistance profile is essential for the effective designing and execution of successful and sustainable vector control interventions as well as for evaluating the impact of insecticides resistance.

In the past, the possibility of generating a large number of F_1 progeny from small numbers of field collected mosquito for molecular characterization constituted a major hindrance for colonizing *An. funestus* in the lab. However, this limitation has been resolved by the invention of the forced egg laying method[14]. An easy approach to addressing this barrier is by collecting indoor resting blood fed female mosquitoes and putting each of them in a confined 1.5-ml tube to forcefully lay eggs. This method has made feasible the substantial evaluation of the susceptibility profile of this mosquito species population against different classes of insecticide. Nevertheless, for

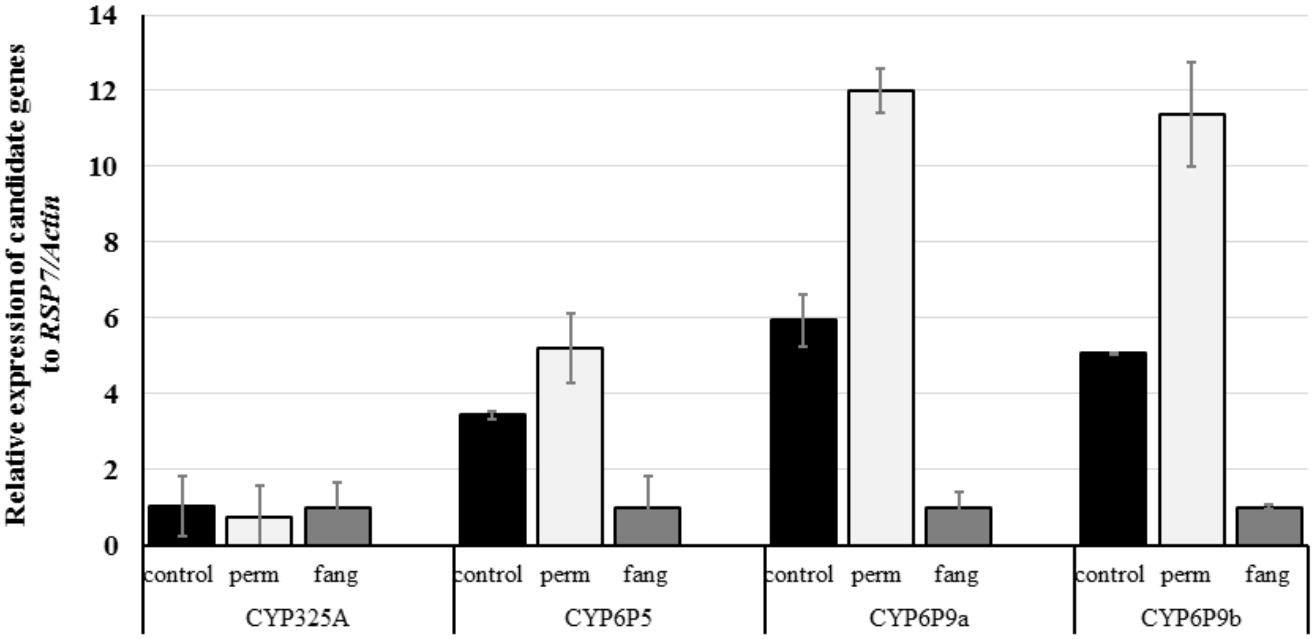

Figure 5. Differential expression by qRT-PCR of the major Cytochrome P450 genes (CYP325A, CYP6P5, CYP6P9a and CYP6P9b) in *An. funestus* s.s. in Elende compared with the susceptible *A. funestus* s.s. strain FANG. Error bars represent standard errors of the mean.

experiments involving F_1 adults to be informative, it is cardinal that, the offspring obtained by this technique should not be bias and family isolation effects must be reduced such that the progeny are typical of the overall population. In this regard, pooled egg batches were reared together and the F_1 adults were randomly combined in cages for the various assays.

With the possibility of generating a large number of progenies from field collected female *An. funestus* and *An. gambiae* mosquitoes, this study therefore characterized the principal malaria vectors in a peri-urban setting within the forested region of Cameroon, located 2 km away from the Yaoundé -Nsimalen International airport.

Mosquito species composition in Elende

From the nine species of *An. funestus* s.l. group described, *An. funestus* s.s., *An. rivulorum* and *An. vaneedeni* were detected in Elende, with abundance of 98.34%, 0.83% and 0.83% respectively. This result is similar to a study conducted in Tibati[35] and Gounougou (northern region of Cameroon), where *An. funestus* s.s. accounted for 99.50% of the species collected and *Anopheles leesoni* was 0.50%[9]. Likewise, the result reflects the species abundance in a published study conducted in Mibellon (Cameroon), where *An. funestus* s.s. was the only dominant vector found within the group[11]. The superiority of *An. funestus* s.s. was also reported in Kpome-Benin (West Africa)[36]. Since this study was done at a single point in time, we cannot exclude the presence of other Anopheline species. More so, the study may have limited the collection of outdoor resting members of the group, since mosquito sampling was concentrated indoors. However, this differs with the distribution of members of this group observed in eastern and southern regions of Africa where several member species were collected indoors. For example, *An. parensis*, *An. leesoni* and *An. rivulorum* were found in higher densities indoors in a study in Uganda and southern Africa[37–41].

The dominance of *An. funestus* s.s. within the *An. funestus* group in this locality further confirms the extremely anthropophilic and endophilic nature of this species which is highly involved in the transmission of human malaria. This result supports the broad geographical distribution of *An. funestus* s.s. in Cameroon where it stands as a major malaria vector[11]. Nonetheless, further studies are required to determine the blood meal source of the major *Anopheles* vectors and their species abundance in outdoor settings in order to have an overview of the vectorial capacity and malaria transmission dynamics in this locality.

Regarding the *An. gambiae* complex, *An. gambiae* was the only species found. This result is similar to previous studies demonstrating that *An. gambiae* s.s. was the major species in rural and semi-rural areas of the Centre and Littoral regions in Cameroon, particularly in Yaounde and Douala[42–44].

Roles of both vectors in malaria transmission in forested areas

This study confirms the role of *An. funestus* s.s. and *An. gambiae* s.s. in malaria transmission in this locality with sporozoite infection rate of 6% and 5% respectively. This result is similar to *An. funestus* s.s. sporozoite infectivity rate in Mibellon (5%) but higher than in Obout (3.2%) and Tibati (2.94%). Due to the low number of the field-collected *An. gambiae* s.s. during

the study period, infection with *Plasmodium* (5%) was similar to *An. funestus* s.s. (6%). Also, this rate is similar to previous results in Cameroon[10]. Because the location of Elende is close to the Nsimalen-International Airport and to the city of Yaoundé, efforts should be made to reduce the malaria transmission in this locality to avoid it constitute a reservoir for transmission in the city particularly as it was recently shown that mosquitoes can fly over long distances[12].

Multiple and high insecticide resistance in both major vectors constitutes a barrier for vector control in forested areas

Insecticide resistance profile of *An. funestus* s.s. in Elende locality is similar to previous studies in Cameroon documented for this species, where resistance against all pyrethroids and full susceptibility to organophosphates was observed[11]. The multiple insecticide resistance patterns observed in the *An. funestus* s.s. population to pyrethroids and DDT in Elende corresponds to the trends observed in Gounougou (2012)[9] and higher than in Obout (2016)[45] but lower than in Tibati (2018)[35]. Moreover, the high resistance pattern of *An. funestus* s.s. to pyrethroids observed in this locality is similar to that observed in Mibellon (2017)[11]. This result brings to attention the fact that resistance in *An. funestus* s.s. is pervasive in Cameroon and constitute a threat for operational insecticide-based vector control tools directed at this species. In Cameroon, the massive deployment of LLINs implemented by the Cameroonian Government in the past years has likely contributed to a rapid rise of pyrethroid resistance in *An. funestus* s.s. vector. Moreover, Elende is also located in an area where farming is widely practiced, and agricultural application of pesticides for crop protection apparently imposes a selective pressure that further pilots the increase in resistance level. Similarly, this same pattern of high pyrethroid resistance in *An. funestus* s.s. was observed in Southern Africa in Malawi[46] and Mozambique[47]; the East African region including Uganda[48]; and West Africa in Ghana[49], Benin[36] and Nigeria[50].

The full reversal to susceptibility observed after PBO exposure to permethrin and deltamethrin, implies that cytochrome P450 genes are playing a notable role in the resistance mechanisms. This increasingly higher resistance to pyrethroids poses a remarkable challenge for malaria control programs in Cameroon and necessitates the urgent implementation of insecticide resistance management strategies so as to prevent failure of future programs directed at scaling-up distribution campaigns of pyrethroid impregnated LLINs.

Extremely high levels of resistance to several classes of insecticides, including organochlorine, pyrethroid and carbamate, were also noticed in the *An. gambiae* s.s. population from Elende. Moreover, the intense resistance of *An. gambiae* s.s. to 5x and 10x concentration of permethrin and deltamethrin each suggests that the resistance is elevated in this population. This elevated resistance in *An. gambiae* s.s. corresponds with the high level of resistance reported in this species across various sites in Cameroon[6–8,35]. Furthermore, the reduced susceptibility observed against the organophosphates (malathion, fenithrothion and pirimiphos-methyl) in *An. gambiae* s.s. is an indication of possible cross resistance with the carbamates since both insecticides class act on the same nervous system target site. In this regard, carbamates insecticide should be excluded as a replacement to pyrethroid for IRS as this will further select the spread of the resistant allele within the species population of this locality.

The resistance in *An. gambiae* s.s. was higher compared to *An. funestus* s.s. for almost all the insecticides during this time interval, suggesting a substantial selection pressure acting on *An. gambiae* s.s. This could be as a result of environmental and genetic selection of resistance from breeding sites polluted with pesticides used for crop protection. Since *An. gambiae* s.s. temporal breeding sites are often located nearby areas of crop cultivation, the selection would be enormous in this species compared to *An. funestus* s.s. However, the sample size of field collected *An. gambiae* that laid eggs was low (n=20) and this may have limited the ability to capture the full susceptibility profile of this population to insecticides. Nevertheless, the consistency of the resistance profile with notably the fixation of kdr and RDL in these mosquito population supports the findings that the profile presented in this study could reflect the ongoing resistance pattern.

Bio efficacy of LLINs in cone assays

Freely distributed LLINs by the National Malaria Contol Programme (NMCP) constitute the central malaria vector control intervention in Elende. The dramatic drop in potency of these solely impregnated pyrethroid nets is comparable to cases reported in other localities in Cameroon[11] and Africa[36,47]. Resistance to pyrethroids in this species is linked with a marked decline in efficacy to all pyrethroid only LLINs as demonstrated by the diminishing mortality rates against PermaNet 2.0 (<10%) and Olyset net (<25%). Conversely, PBO-based nets demonstrated a greater efficacy with the highest reported by both PermaNet 3.0 top and Olyset plus scoring 100% mortality. This indicates that cytochrome P450 genes are probably propelling pyrethroid resistance in this locality. The higher mortality rate observed with PBO-based nets suggest that these synergist nets including Olyset Plus and PermaNet 3.0 (roof) should be regarded as a substitution to pyrethroid-only nets in areas of increasing resistance fueled by metabolic mechanisms particularly for cytochrome P450s as it is the situation for *An. funestus* s.s.[29].

Elevated metabolic resistance in *An. funestus* differs with high levels of knockdown resistance in *An. gambiae*

The full susceptibility noticed for pyrethroids in *An. funestus* s.s. after first exposure to PBO points out that metabolic resistance mediated by cytochrome P450s is the main mechanism[51]. This is linked to previous studies which confirm the absence of *kdr* target site sensitivity mutation in this species in Cameroon[9] and across Africa[52]. In the absence of voltage-gated sodium channel knockdown resistance mutations in *An. funestus* s.s.[52], this study demonstrated that pyrethroid resistance in Elende populations of *An. funestus* s.s. is possibly steered by metabolic resistance machinery. Overall, the role of metabolic resistance is apparent by the marked up-regulation of

the three P450 genes (*CYP6P5*, *CYP6P9a* and *CYP6P9b*) already reported as essential genes conferring pyrethroid resistance in *An. funestus* s.s. populations across Africa[30].

The absence of the *CYP6P9a* resistance allele in *An. funestus* s.s. population from Elende corresponds to the study by Weedall *et al.*[26]. This confirms the fact that this mutation, fixed in mosquitoes from southern Africa is not yet present in mosquitoes from Central/West Africa[26].

Cross-resistance to DDT and pyrethroids has been demonstrated to be conferred by GSTe2. In relation to this, the frequency of the L119F-GSTe2-resistant allele in the Elende field population (48%) is higher than in Mibellon (28%), Tibati (10.2%) and lower than in Gounougou (52%). Similarly, across Africa, the frequency of the DDT resistance marker was closer to that observed in Democratic Republic of Congo[53] and Ghana[49]; higher than the frequency reported in eastern part of Africa, Uganda[37,38] although lower to studies in Benin[36].

The frequency of the 296S-RDL-resistant allele is 100%, which is higher than in the northern region, particularly in Mibellon (9.7%), Gounougou (14.6%) and Tibati (0.4%). However, this result is similar to mortality rate recorded in *An. funestus* s.s. from Obout that exhibited very high level of resistance to dieldrin (4.35% mortality rate)[45]. This high frequency could be as a result of strong resistance selection due to environmental persistence of insecticide residues since its withdrawal from public and agricultural use in Cameroon.

The elevated resistance levels to pyrethroids in *An. gambiae* s.s. accords with the increased frequency of the 1014F *kdr* allele (94.9%). This correlates with past studies done in Africa where high pyrethroid resistance in *An. gambiae* s.l. has been coupled with almost fixed *kdr* allele in the population, as recently reported in DR Congo[53], or earlier in Côte d'Ivoire[54]. Consistent with previous research performed in other parts of Cameroon[8,35], this study found elevated frequencies of the *kdr* mutation in An. *gambiae* s.s. population in Elende that has almost reached fixation.

The very low frequency of the 1014S *kdr* allele in Elende (1.28%) is in parallel to earlier reports across Cameroon exhibiting that this target site resistance mutation, originally discovered in East Africa, is gradually spreading to Central and West Africa although still at very low occurrence[55].

The presence of the 119S *ace-1* mutation in *An. gambiae* s.s. population is in line with the reduced susceptibility observed in this population to carbamates and organophosphates[34,56,57]. The use of carbamates and organophosphates may be regarded as an alternative for the management of this highly insecticide resistant vector population although the detection of *Ace-1* is also a cause of concern.

Similar studies involving large sample sizes should be conducted across different ecological settings in Cameroon to establish the epidemiological and entomological parameters of malaria transmission and investigate the resistance profile of malaria vectors to existing insecticides. Specifically, a longitudinal survey would obviously provide useful and interesting information on the seasonal species composition and abundance, in-depth knowledge on the biology of each species, mosquito host-seeking and resting preferences, pattern of insecticide resistance,frequency of insecticide resistance genes and the role played by *Anopheles* vectors in malaria transmission in this locality over time. Data produced from such future studies will be relevant in generating additional significant information required to strengthen malaria control.

Conclusion

This study reports the preliminary characterization and resistance profile of endophilic malaria vectors *An. funestus* and *An. gambiae* in Elende locality, situated close to a port of entry in Yaounde, the capital city of Cameroon. The significant *Plasmodium* sporozoite infection rate alongside the resistance to multiple insecticide classes observed in these vector populations highlights the challenges that public health vector control programs encounter in sustaining the regular effectiveness of contemporary insecticide-based control interventions aimed at reducing malaria transmission in forested areas. More particularly, the baseline resistance observed against the carbamates and possible resistance against the organophosphates constitutes a major concern for IRS; while suggesting the susceptibility evaluation of *Anopheles* malaria vectors in this locality to neonicotinoids and pyrrole insecticides in preparation for indoor residual spraying campaigns with novel insecticide ingredients. Also, this study further provides operational evidence to National Malaria Control Programs for a shift from mass distribution of pyrethroid-only LLINs to second-generation bed nets (containing synergist) in areas where high resistance is driven by metabolic mechanisms notably cytochrome P450s.

Data availability

Underlying data

Open Science Framework: Elevated Plasmodium sporozoite infection and multiple insecticide resistance in the principal malaria vectors Anopheles funestus and Anopheles gambiae in a forested locality close to the airport of Yaoundé, the Capital city of Cameroon. https://doi.org/10.17605/OSF.IO/XN68J[34].

This project contains the following underlying data:

- 1-Raw output Ct values_qRT-PCR experiment_data_ including housekeeping genes (XLSX). (Raw Ct values generated during qRT-PCR experiments.)
- 2-Taqman derived_*Plasmodium* infection rates_*An. funestus* and *An. gambiae*_Elende (XLSX). (*Plasmodium* mosquito infection rates, measured via TaqMan assay.)
- 3-*An. funestus* and *An. gambiae*_PCR species identification (XLSX). (Mosquito species identification data, performed using PCR.)
- 4-Pyrethroid exposure experiments & WHO insecticide susceptibility & PBO synergist assay (XLSX). (Raw insecticide susceptibility mortality data.)

- 5-Data generated from cone assay (XLSX). (Raw mortality data generated via cone assays.)
- 6-Data generated for resistance marker genotyping experiments (XLSX). (Raw qRT-PCR resistance genotyping data.)

The *An. funestus* s.s. ITS2 nucleotide sequences have been deposited in GenBank with accession numbers: MT991011 to MT991016.

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 13 November 2020

https://doi.org/10.21956/wellcomeopenres.17917.r41288

✔ **Louisa A. Messenger**
Department of Disease Control, Faculty of Infectious Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

Many thanks to the authors for addressing all of my comments and concerns.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Medical entomology, population genetics, insecticide resistance, molecular biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 18 August 2020

https://doi.org/10.21956/wellcomeopenres.17349.r39209

✔ **Mamadou Ousmane Ndiath**
Disease Control & Elimination Theme, Medical Research Council Unit, London School of Hygiene and Tropical Medicine, Fajara, The Gambia

Jessy Goupeyou-Youmsi

MAC Communicable Diseases Action Center, , College of Medicine - University of Malawi, Blantyre, Malawi

General comments
The authors characterized the species composition of *Anopheles* mosquitoes at Elende in Cameroon, investigated the insecticide resistance profile (using the four classes of insecticides) of two mosquito species (*An. funestus* s.s. and *An. gambiae* s.s) that are major malaria vectors in Cameroon, and evaluated the sporozoite carriage rate in these two mosquito species. This research study is relevant to several fields including entomology and malaria transmission. The results of this study will help strengthen current vector control interventions for malaria control in Cameroon. The authors reviewed the existing literature adequately and provided the source data underlying the results available to ensure full reproducibility. However, the study design is partly appropriate, leading to some conclusions which are not adequately supported by the results. See major and minor comments below.

- **Major comments**: The sample size is low, both for the insecticide susceptibility test and molecular biology. Based on the underlying data raw, only one replicate was done for the PBO synergist assays, while according to the WHO test procedures for insecticide resistance monitoring in malaria vector mosquitoes, this should be at least 2 replicates (not to be confused with the number of test tubes per treatment).

- **Minor comments**: Indoor collection of mosquitoes only does not allow to characterize mosquito species composition. The good point is that the authors highlighted this limitation in the discussion. We suggest the authors to remove this as an objective since the goal was to collect bloodfed mosquitoes to produce the F1 progeny for insecticide resistance assays. We also suggest the authors to rephrase the expression “high *Plasmodium* infection rate” throughout the manuscript for the reason we gave in the following paragraph.

Title

- We suggest authors to remove the word “Elevated” in the title and to rephrase it, since the results of *Plasmodium* infection rate are not strong enough to support the affirmation “Elevated *Plasmodium* sporozoite infection”. Indeed, (1) the sample size is very low (150 for *An. funestus* and 39 for *An gambiae*), (2) mosquitoes analyzed were bloodfed (=> more chance of contamination) and (3) the PCR assay was not *Plasmodium* stage-specific.

Abstract

- It will help the reader to specify at the Methods section the insecticides used or at least that the four classes of insecticides were used.

- Add the word “THE” in the following sentence: “*Anopheles funestus* s.s. was THE most…”.

- It would be good to use either the name of the insecticide or the insecticide class when giving results.

- The term “high *Plasmodium* sporozoite rate” is a very strong statement given the results.

Introduction

- In the sentence “…the insecticide resistance profiles of *An. funestus* s.s. and *An. gambiae* s.s. population collected…”, add S to the word population.

Methods

- WHO insecticide susceptibility test: clarify why the resistance intensity to pyrethroids was studied only for *An. gambiae.*

- PBO synergist assay:
  - How many replicates were done?
  - In the sentence "...exposure to permethrin (0.75%) and deltamethrin (0.05%) for another 1 h interval.", replace "and" by OR and "interval" by EXPOSURE.
- Assessment of bed net efficacy using cone assay: "Due to the insufficiency of *Anopheles gambiae* s.s.,...", replace "insufficiency by LOW ABUNDANCE.

Results

Plasmodium infection rate:

- About the TaqMan Assay, what was the number of cycles for denaturation? (give the PCR conditions if different from what of Bass et al, 2008). This an important parameter to take into account as that will help to choose the Ct beyond which all amplified samples will be considered as negative. Looking at the underlying data raw, all samples with amplification signal after the 39^{th} cycle should have been considered as negative. The Ct value 37.39 of the positive control OVM is quite high (replicate 59 in tab 2).
- In the underlying data raw, we counted 9 (not 10) *An. funestus* samples positive to *Plasmodium* (7 *P. falciparum* and 2 OVM). Please check again.
- About the sentence "The *Plasmodium* infected mosquitoes revealed by TaqMan was further confirmed with nested PCR.", rephrase it like this: *Plasmodium* infected mosquitoes revealed by TaqMan were further tested with nested PCR.
- Insecticide susceptibility assays: Indicate the total number of mosquitoes tested per treatment (insecticide) for both *An. funestus* and *An. gambiae*. Also add this information in the figure 2.
- PBO synergist assays
  - How many replicates were done?
  - How many mosquitoes tested per treatment? Indicate this in the figure 3.
- In the underlying data raw (WHO susceptibility test and cone bioassays), check again start and end times so that it matches 1H.

Discussion

- Taking into account the information given by the authors about the study area, it seems like Elende suits better a peri-urban setting.
- "Due to the low number of the field-collected *An. gambiae* s.s. during the study period, infection with *Plasmodium* (5%) was lower compared to *An. funestus* s.s. (6.6%).": the authors should support this statement with statistical analysis.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 07 Oct 2020

Nkemngo Francis Nongley, Centre for Research in Infectious Diseases (CRID), Yaounde, Cameroon

Mamadou Ousmane Ndiath
Disease Control & Elimination Theme, Medical Research Council Unit, London School of Hygiene and Tropical Medicine, Fajara, The Gambia
Jessy Goupeyou-Youmsi
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General comments
The authors characterized the species composition of Anopheles mosquitoes at Elende in Cameroon, investigated the insecticide resistance profile (using the four classes of insecticides) of two mosquito species (*An. funestus s.s.* and *An. gambiae s.s*) that are major malaria vectors in
Cameroon, and evaluated the sporozoite carriage rate in these two mosquito species. This research study is relevant to several fields including entomology and malaria transmission. The
results of this study will help strengthen current vector control interventions for malaria control in Cameroon. The authors reviewed the existing literature adequately and provided the source data underlying the results available to ensure full reproducibility. However, the study design is partly appropriate, leading to some conclusions which are not adequately supported by the results. See major and minor comments below.

Comment 1:
Major comments: The sample size is low, both for the insecticide susceptibility test and

molecular biology. Based on the underlying data raw, only one replicate was done for the PBO synergist assays, while according to the WHO test procedures for insecticide resistance monitoring in malaria vector mosquitoes, this should be at least 2 replicates (not to be confused with the number of test tubes per treatment).

Response:

- We thank the reviewer for this comment. We are sorry if the explanation of the samples used was not clear enough. However, we will like to emphasize that for all tests done for *An. funestus*, we performed 4 replicates with around 80-100 mosquitoes in total, and for PBO synergist assays we did the same which is enough to capture the susceptibility profile in this population. This is now better explained in the text notably for PBO wherein a total of 489 mosquitoes were in fact used including the controls as we also indicated a similar response to a query from the other reviewer.

Comment 2:

Minor comments: Indoor collection of mosquitoes only does not allow to characterize mosquito species composition. The good point is that the authors highlighted this limitation in the discussion. We suggest the authors to remove this as an objective since the goal was to collect blood-fed mosquitoes to produce the F1 progeny for insecticide resistance assays.

Response:

- We agree indoor collection does not substantially allow for characterization of mosquito species composition; but it does provide a basis for identifying the endophilic vectors which constitute a great arm for indoor malaria transmission. We have revised this objective as highlighted by the authors in the paragraph before the method section as: This included the characterization of the **endophilic mosquito species** composition, investigation of the insecticide resistance profiles of *An. funestus* s.s. and *An. gambiae* s.s. populations collected in Elende in 2019.

Comment 3:

We also suggest the authors to rephrase the expression “high *Plasmodium* infection rate” throughout the manuscript for the reason we gave in the following paragraph.

Response:

- We have now removed high as suggested by the reviewer.

Comment 4:

◯Title: We suggest authors to remove the word “Elevated” in the title and to rephrase it, since the results of *Plasmodium* infection rate are not strong enough to support the affirmation

“Elevated Plasmodium sporozoite infection”. Indeed, (1) the sample size is very low (150 for *An. funestus* and 39 for *An gambiae*), (2) mosquitoes analyzed were bloodfed (=> more chance of contamination) and (3) the PCR assay was not Plasmodium stage-specific.

Response:

- The title has been revised accordingly and is as follows “Multiple insecticide resistance and *Plasmodium* infection in the principal malaria vectors *Anopheles funestus* and *Anopheles gambiae* in a forested locality close to the Yaoundé airport, Cameroon”

Comment 5:

◯ Abstract: It will help the reader to specify at the Methods section the insecticides used or at least that the four classes of insecticides were used.

Response:

- We thank the reviewer for this comment. We have revised the sentence to include the fact that the four classes of insecticides were used. Thus, the sentence has been modified to "Bioassays were performed to assess resistance profile to the four insecticides classes"

Comment 6:

◯ Add the word "THE" in the following sentence: "*Anopheles funestus s.s*. was THE most...". It would be good to use either the name of the insecticide or the insecticide class when giving results.

Response:

- We sincerely thank the reviewer for this detail comments. "The" has been added to the sentence and the various pyrethroid insecticides used have been included in the sentence which now reads "Both species exhibited high levels of resistance to the pyrethroids, permethrin and deltamethrin (<40% mortality)".

Comment 7:

◯ The term "high *Plasmodium* sporozoite rate" is a very strong statement given the results.

Response: We agree and high has been removed.

Comment 8:

Introduction

In the sentence "...the insecticide resistance profiles of *An. funestus s.s.* and *An. gambiae s.s.* population collected...", add S to the word population.

Response:

- Thank you for the comment. "S" has been added.

Comment 9:

◯Methods

WHO insecticide susceptibility test: clarify why the resistance intensity to pyrethroids was studied only for *An. gambiae*.

Response:

- We did not have sufficient numbers of F1 *An. funestus* progeny to do the resistance intensity bioassay. We plan to perform intensity bioassay on An. funestus in future studies.

Comment 10:

◯ PBO synergist assay:

◯ How many replicates were done?

Response:

- The following replicates were conducted for PBO synergist assay:

Permethrin 0.75%: 04 replicates
PBO 4% + Permethrin 0.75%: 04 replicates
Deltamethrin 0.05%: 04 replicates
PBO 4% + Deltamethrin 0.05%: 04 replicates
PB0 4%: 03 replicates
Control: 03 replicate

Comment 11:

In the sentence "...exposure to permethrin (0.75%) and deltamethrin (0.05%) for

another 1 h interval.", replace "and" by OR and "interval" by EXPOSURE.
Response: Thanks for this comment. These changes have been made.
Comment 12:
◯Assessment of bed net efficacy using cone assay: "Due to the insufficiency of Anopheles *gambiae* s.s.,...", replace "insufficiency by LOW ABUNDANCE.

Response:

- Thank you. This change has been made.

Comment 13:
◯Results
Plasmodium infection rate:
About the TaqMan Assay, what was the number of cycles for denaturation? (give the PCR conditions if different from what of Bass et al, 2008). This an important parameter to take into account as that will help to choose the Ct beyond which all amplified samples will be considered as negative. Looking at the underlying data raw, all samples with amplification signal after the 39th cycle should have been considered as negative. The Ct value 37.39 of the positive control OVM is quite high (replicate 59 in tab 2).
Response:

- Basically, the PCR conditions were: segment 1 [1 cycle at 95^0C/10mins] and segment 2 [40 cycles at 92^0C/15s and 60^0C/1min]. We thank the reviewer for bringing this point up. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). As such, high Ct values can also mean lower (and even too little) amounts of the target nucleic acid and not necessarily a negative sample. Indeed, the high Ct value of the P. OVM positive control indicates that the *Plasmodium* parasite DNA was present in little amount. This P. OVM control was further confirmed with nested PCR where a band size of 144bp was observed indicative of *P. malariae*.

Comment 14:
◯In the underlying data raw, we counted 9 (not 10) *An. funestus* samples positive to *Plasmodium* (7 P*. falciparum* and 2 OVM). Please check again.
Response:

- We sincerely appreciate this detail comment from the reviewer. We double-checked again from the original data and agree with your remark. In line, the *Plasmodium* infection rate for *An. funestus* was 6% (09/150). We have corrected the infection rate and associated statistics in the revised version of the manuscript.

Comment 15:
◯About the sentence "The Plasmodium infected mosquitoes revealed by TaqMan was further
confirmed with nested PCR.", rephrase it like this: *Plasmodium* infected mosquitoes revealed by TaqMan were further tested with nested PCR.

Response:

- We thank the reviewer for noting this. As mentioned, the sentence has been rephrased to: "Plasmodium infected mosquitoes revealed by TaqMan were further tested with nested PCR" in the revised version of the manuscript.

Comment 16:
◯ Insecticide susceptibility assays: Indicate the total number of mosquitoes tested per treatment (insecticide) for both *An. funestus* and *An. gambiae*. Also add this information in PBO synergist assays
◯ How many replicates were done?
◯ How many mosquitoes tested per treatment? Indicate this in the figure 3.
◯ In the underlying data raw (WHO susceptibility test and cone bioassays), check again start and end times so that it matches 1H.
Response:
- Thank you for this remark. The total number of mosquitoes tested per insecticide for both vectors have been included in the revised version of the manuscript. Similarly, the same revision has been done for the PBO assay in the text displaying fig 3. Also, the underlying raw data has been re-checked.

Comment 17:
◯Discussion
Taking into account the information given by the authors about the study area, it seems like Elende suits better a peri-urban setting.
Response:
- We agree with the reviewer. We have modified the suggestion accordingly.

Comment 18:
◯ "Due to the low number of the field-collected *An. gambiae s.s*. during the study period, infection with *Plasmodium* (5%) was lower compared to *An. funestus s.s.* (6.6%).": the authors should support this statement with statistical analysis
Response:
- Thank you for this comment for which we agree. We have modified the sentence of this comparison.

Competing Interests: None

Reviewer Report 23 July 2020

https://doi.org/10.21956/wellcomeopenres.17349.r39478

?

Louisa A. Messenger
Department of Disease Control, Faculty of Infectious Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

This article characterizes malaria vector dynamics (species composition, insecticide resistance phenotypic profiles and mechanisms and Plasmodium infection) in a forested region of Cameroon, where little previously data have been reported previously. This article provides information to

guide local prospective malaria vector control measures and resistance management strategies.

My main concern with this study is the very small number of mosquitoes. The mosquito collections were only performed for two days – this is a very short period of time. Indoor mosquito collections can certainly be more difficult and not as productive as other sampling methods, but the small sample sizes in this study do limit the conclusions the authors can make considerably.

Minor comments:

- In the methods, “study area and mosquito collection” section, instead of speculating that the marshy lands are “accompanied by an increased malaria transmission”, can you please provide some data to support this?

- In the methods, “study area and mosquito collection” section, how many houses were sampled for mosquito collections to give an idea of whether you likely just sampled mosquito siblings or were able to gather more-like population-level data for this area.

- In the methods “study area and mosquito collection” section, can you specify which pyrethroid LLINs have been distributed, to enable better interpretation of the resistance data.

- In the methods “WHO insecticide susceptibility bioassays” section, why was DDT and fenitrothion tested? I just wonder if it might have been a better use of your mosquitoes to test *An. funestus* s.l. with pirimiphos-methyl as well, given it is a potential IRS candidate in Cameroon.

- In the methods “WHO insecticide susceptibility bioassays” section, did you interpret the 5X and 10X diagnostic results with reference to the latest WHO guidelines? I.e. at 5X <98% mortality indicates moderate to high intensity resistance and at 10X <98% mortality indicates high intensity resistance.

- In methods “molecular species identification” section, have the authors deposited the ITS2 sequences in GenBank, or another open-access data repository (I could not easily find it among the supporting data files)? If not, can they please do so? It would be interesting to see if there was some sequence variability which might explain the proportion of non-amplifying *An. funestus* s.s. using the cocktail PCR.

- In the genotyping methods sections, why was genotyping performed on F0 mosquitoes, when to directly relate individual mosquito phenotype to its genotype it should have been performed with F1 mosquitoes? This was done with the transcription profiling. Is this a simple typing mistake?

- In the “transcription profiling of candidate resistance associated genes”, what do you mean by “F1 females each that recovered after 1 h exposure”? Do you mean mosquitoes which survived the 1 hr exposure and can be classified as “resistant” (as you do subsequently) or do you mean those that were knocked-down during exposure and then later revived after the test? As these are two distinct phenotypic populations, please clarify.

- In the results “species identification” section, the mosquito populations under evaluation,

with founding population sizes of 196 *An. funestus* s.s. and 20 *An. gambiae* s.s., are likely to be very genetically restricted/highly related, which limits the applicability and usefulness of these data. Similarly, the number of mosquitoes screened for malaria and genotyped for resistance mutations was really low. This point is touched upon in the discussion but it is quite likely that this study is biased by familial isolation effects.

- In the results "insecticide susceptibility assays" or in the figures, can the authors please specify the number of mosquitoes tested per dose? According to the WHO guidelines this should be approximately 100 mosquitoes per dose (plus appropriate controls), but the authors report screening 598 mosquitoes for 7 insecticides, so I assume less replicates were performed? Especially for PBO – I am not sure how you managed to run negative and positive controls, PBO pre-exposures and pyrethroid-only exposures in parallel, using only 169 mosquitoes?

- Given the number of experimental limitations, including sample sizes and collection duration, it would be good to see the authors acknowledge these more openly in the discussion please.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Medical entomology, population genetics, insecticide resistance, molecular biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 07 Oct 2020

Nkemngo Francis Nongley, Centre for Research in Infectious Diseases (CRID), Yaounde, Cameroon

Louisa A. Messenger
Department of Disease Control, Faculty of Infectious Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

This article characterizes malaria vector dynamics (species composition, insecticide resistance phenotypic profiles and mechanisms and Plasmodium infection) in a forested region of Cameroon, where little previously data have been reported previously. This article provides information to guide local prospective malaria vector control measures and resistance management strategies.

Query 1:
My main concern with this study is the very small number of mosquitoes. The mosquito collections were only performed for two days – this is a very short period of time. Indoor mosquito collections can certainly be more difficult than and not as productive as other sampling methods, but the small sample sizes in this study do limit the conclusions the authors can make considerably.
Response:

- We thank the reviewer for this comment. A total of 196 randomly collected *An. funestus* laid eggs. If we considered that it has been previously shown that the average number of mosquitoes that constitute a population (a deme) is around 50 individuals (Besansky et al 1997), the number of 196 might be sufficient to capture the overall susceptibility profile of this population. For *An. gambiae* a total of 20 did lay eggs and we agree that this is low, but this is the maximum we could obtain. This limitation for *An. gambiae* is now highlighted in the manuscript as follows: “However, the sample size of field collected *An. gambiae* that laid eggs was low (n=20) and this may have limited the ability to capture the full susceptibility profile of this population to insecticides. Nevertheless, the consistency of the resistance profile with notably the near fixation kdr and that of RDL in these mosquito population supports the findings that the profile presented in this study could reflect the ongoing resistance pattern”.

Query 2:
Minor comments:
In the methods, “study area and mosquito collection” section, instead of speculating that the
marshy lands are “accompanied by an increased malaria transmission”, can you please provide some data to support this?
Response:

- We agree with the reviewer that no data is currently available to support the increase malaria transmission in Elende due to the marshy lands. Therefore, we have deleted the related statement: “accompanied by an increase malaria transmission”.

Query 3:
In the methods, “study area and mosquito collection” section, how many houses were

sampled for mosquito collections to give an idea of whether you likely just sampled mosquito siblings or were able to gather more-like population-level data for this area.

Response:

- The number of houses sampled for mosquito collection has been included in the text as follows: “from 15-20 randomly selected houses to avoid sampling individuals from single female egg batches and to obtain a representative population-level data for this area”.

Query 4:

In the methods “study area and mosquito collection” section, can you specify which pyrethroid LLINs have been distributed, to enable better interpretation of the resistance data.

Response:

- Thank you for this interesting remark. PermaNet 2.0 (Deltamethrin 55mg/m^2) was the pyrethroid LLIN distributed in this locality.

Query 5:

In the methods “WHO insecticide susceptibility bioassays” section, why was DDT and fenitrothion tested? I just wonder if it might have been a better use of your mosquitoes to test An. funestus s.l. with pirimiphos-methyl as well, given it is a potential IRS candidate in Cameroon.

Response:

- We agree that testing pirimiphos-methyl would have been ideal. Unfortunately, this insecticide paper was not available. This will surely be done in the future. We tested DDT and fenitrothion to obtained a profile of resistance against all 4 classes of insecticides.

Query 6:

In the methods “WHO insecticide susceptibility bioassays” section, did you interpret the 5X and 10X diagnostic results with reference to the latest WHO guidelines? I.e. at 5X <98% mortality indicates moderate to high intensity resistance and at 10X <98% mortality indicates high intensity resistance.

Response:

- We thank the reviewer for this comment. We have now clearly indicated that the WHO criteria was used to assess the resistance intensity as shown in the sentence below in the text. “High intensity resistance to 5x and 10x permethrin was observed with a recorded mortality rate of 31.74% and 44.30% respectively which according to the WHO guidelines (WHO, 2016) indicates a high intensity of resistance in Elende.”

Query 7:

In methods “molecular species identification” section, have the authors deposited the ITS2 sequences in GenBank, or another open-access data repository (I could not easily find it among the supporting data files)? If not, can they please do so? It would be interesting to see if there was some sequence variability which might explain the proportion of non-amplifying An. *funestus* s.s. using the cocktail PCR.

Response:

- Thank you for this observation. The ITS2 sequences have been deposited in GenBank with accession numbers: MT991011 to MT991016. These have been included in the text.

Query 8:

In the genotyping methods sections, why was genotyping performed on F0 mosquitoes, when to directly relate individual mosquito phenotype to its genotype it should have been performed with F1 mosquitoes? This was done with the transcription profiling. Is this a simple typing mistake?

Response:

- Genotyping was done for the F0 mosquitoes to obtain the frequency of resistance in the field since they are the operationally relevant exposed population.
- The transcriptional profiling was done of F1 as it is easier to get enough numbers as three pool of 30 mosquitoes alive after performing the bioassay. Also controlling the age of the mosquitoes so as to perform bioassay with mosquitoes aged 3 to 5 days old is complicated with F0 for *An. funestus* as finding their larvae is difficult.

Query 9:

In the “transcription profiling of candidate resistance associated genes”, what do you mean by “F1 females each that recovered after 1 h exposure”? Do you mean mosquitoes which survived the 1 hr exposure and can be classified as “resistant” (as you do subsequently) or do you mean those that were knocked-down during exposure and then later revived after the test? As these are two distinct phenotypic populations, please clarify.

Response:

- Thanks for bringing this up. Here, we are referring to the mosquitoes alive 24 hours post 1hour insecticide exposure.

Query 10:

In the results “species identification” section, the mosquito populations under evaluation, with founding population sizes of 196 An. funestus s.s. and 20 An. gambiae s.s., are likely to be very genetically restricted/highly related, which limits the applicability and usefulness of these data.

Similarly, the number of mosquitoes screened for malaria and genotyped for resistance mutations was really low. This point is touched upon in the discussion but it is quite likely that this study is biased by familial isolation effects.

Response:

- This comment is already addressed above in the major comments section (Query 1). For *An. gambiae*, we highlighted this limitation in the discussion. For *An. funestus* the number of 196 F0 females that laid eggs and thus contributing to the F1 is high enough (based on expected size of a deme) to be representative of the genetic variability in this population and reflects similar studies previously performed on this species for which it is difficult to collect field larvae for such tests.

Query 11:

In the results “insecticide susceptibility assays” or in the figures, can the authors please specify the number of mosquitoes tested per dose? According to the WHO guidelines this should be approximately 100 mosquitoes per dose (plus appropriate controls), but the authors report screening 598 mosquitoes for 7 insecticides, so I assume less replicates were performed?

Response:

- Thank you for this comment. In total, we employed the sum of 638 *An. funestus s.s* F1 for the insecticide susceptibility assay. Unfortunately, due to the low number, we could not perform the test with 100 samples for all insecticides tested. Nevertheless, the results presented provide an indication of the current susceptibility profile. We have included the number of mosquitoes tested per treatment in the manuscript.

Query 12:
Especially for PBO – I am not sure how you managed to run negative and positive controls, PBO pre-exposures and pyrethroid-only exposures in parallel, using only 169 mosquitoes?

Response:
- The positive and negative controls were performed for PBO but with less than 100 mosquitoes and this was done at the same time as the other tests to take advantage of a common negative control.
- Thank you for this comment. Overall, a total of 489 F1 female *An. funestus s.s*. mosquitoes were utilized for the complete PBO synergist assay (which includes parallel and simultaneous exposure of the F1 female offspring mosquito population to: pyrethroid only impregnated papers, PBO only impregnated papers, PBO + pyrethroid exposures and controls). In the PBO synergist assay section of the manuscript, 169 F1 *An. funestus s.s.* mosquitoes referred only to the pyrethroid + PBO exposed not including the pyrethroids only exposed and the controls. When these are included, the total is 489.

Query 13:
Given the number of experimental limitations, including sample sizes and collection duration, it would be good to see the authors acknowledge these more openly in the discussion please.

Response:
The limitation in sample size concerns mainly *An. gambiae* which is now acknowledged in the discussion as suggested by the reviewer.

Competing Interests: None