RESEARCH ARTICLE

REVISED Investigation of the influence of a glutathione S-transferase metabolic resistance to pyrethroids/DDT on mating competitiveness in males of the African malaria vector, *Anopheles funestus* [version 2; peer review: 2 approved, 1 approved with reservations]

Previously titled: Investigation of the influence of a glutathione S-transferase metabolic resistance to pyrethroids/DDT on mating competitiveness in males *Anopheles funestus,* African malaria vector

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Abstract

Background: Metabolic resistance is a serious challenge to current insecticide-based interventions. The extent to which it affects natural populations of mosquitoes including their reproduction ability remains uncharacterised. Here, we investigated the potential impact of the glutathione S-transferase L119F-GSTe2 resistance on the mating competitiveness of male *Anopheles funestus*, in Cameroon.
Methods: Swarms and indoor resting collections took place in March, 2018 in Tibati, Cameroon. WHO tube and cone assays were performed on F_1 mosquitoes from indoor collected females to assess the susceptibility profile of malaria vectors. Mosquitoes mated and unmated males collected in the swarms were genotyped for the L119F metabolic marker to assess its association with mating male competitiveness.
Results: Susceptibility and synergist assays, showed that this population was multiple resistant to pyrethroids, DDT and carbamates, likely driven by metabolic resistance mechanisms. Cone assays revealed a reduced efficacy of standard pyrethroid-nets (Olyset and PermaNet 2.0) with low mortality (<25%) whereas synergist PBO-Nets (Olyset Plus and PermaNet 3.0) retained greater efficacy with higher mortality (>80%). The L119F-GSTe2 mutation, conferring pyrethroid/DDT resistance, was detected in this *An. funestus* population at a frequency of 28.8%. In addition, a total of 15 mating swarms were identified and 21 *An. funestus* couples were isolated from those swarms. A comparative

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genotyping of the L119F-GSTe2 mutation between mated and unmated males revealed that heterozygote males 119L/F-RS were less able to mate than homozygote susceptible (OR=7.2, P<0.0001). Surprisingly, heterozygote mosquitoes were also less able to mate than homozygote resistant (OR=4.2, P=0.010) suggesting the presence of a heterozygote disadvantage effect. Overall, mosquitoes bearing the L119-S susceptible allele were significantly more able to mate than those with 119F-R resistant allele (OR=2.1, P=0.03). **Conclusion:** This study provides preliminary evidences that metabolic resistance potentially exerts a fitness cost on mating competiveness in resistant mosquitoes.

Keywords
Malaria, insecticides, metabolic resistance, Glutathione S-transferase, Anopheles funestus, mating competitiveness

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Any reports and responses or comments on the article can be found at the end of the article.

REVISED **Amendments from Version 1**

The main change in this version of the manuscript is only on the result of *Plasmodium* infection in *An. gambiae s.l.* Compared to the first version where we presented the infection rate in both *An. gambiae* and *An. coluzii* combined, here the result is presented for each species. In addition we have corrected some English mistakes as suggested by the reviewer.

See referee reports

Background

Despite significant reduction of malaria burden in the past decade, this disease remains a major public health concern in Africa. Recent reports of increase cases of malaria[1] is a further indication that more is needed to control this disease. The scale up of vector control measures, in particular long-lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS), has been the main driver of this reduction of malaria burden with about 78% of all gains achieved since 2000 attributed to these methods[2]. However, resistance is spreading in malaria vectors in Africa including *Anopheles funestus* for the four classes of insecticides used in public health, compromising the effectiveness of these interventions[3]. Providing adequate information about the mechanisms of resistance and more importantly its impact on key traits of mosquito biology, ecology and behaviour such as their mating ability in the presence of resistance can help in planning and implementing suitable insecticide resistance management strategies.

Insecticide resistance management strategies including rotation of insecticide rely on the assumption that insecticide resistance alleles are very often detrimental in the absence of insecticide selection pressure[4,5]. The adaptive allele in this case might be associated with modification of physiological processes or resource availability[6] which can lead to decreased performance and fitness disadvantage of resistant mosquitoes[7–9] and therefore, a reversal to susceptibility is expected in the absence of selection pressure from the specific insecticide. However, little is currently known on such fitness costs in field populations of malaria vectors notably for metabolic resistance mechanism because of a lack of DNA-based markers. A previous study using a laboratory strains of *An. gambiae*, demonstrated fewer copulations in dieldrin resistant males when compared with their susceptible counterparts[6]. Berticat *et al.* demonstrated also the disadvantage in competitive mating ability of *Culex pipiens* males with the target-site resistance *Ace1R* genotype, when compared with susceptible males, pointing to its potential impact on the spread and persistence of resistant alleles. In contrast, for malathion resistance in the beetle *Tribolium castaneum* it was noticed that resistance enhanced male reproductive success. If this last case is observed in malaria vectors, it will be a great concern for control program as it will prevent the implementation of resistance management strategies based on the rotation of insecticides. Currently, there is little information on the impact of metabolic insecticide resistance on the mating ability of natural populations of major malaria vectors in Africa. So far the only study on this topic reported a lack of impact of metabolic resistance on male competitiveness of *An. gambiae* field population in Burkina Faso[10]. The study assessed only the global transcription profiling of mated and unmated mosquitoes since a lack of DNA marker for metabolic resistance prevented a direct genotyping correlation with mating status. However, recent progress made in elucidating the molecular basis of metabolic resistance had identified a single amino acid change (L119F) in the glutathione S-transferase epsilon 2 (GSTe2) conferring pyrethroid/DDT resistance in *An. funestus*[11]. This new marker now provide the opportunity to directly investigate the impact of metabolic resistance on mating male competitiveness. However, assessing the impact of resistance on the mating of malaria vectors through swarm's collection in natural populations of mosquitoes requires a good knowledge of the mating places and also the mating behaviour of these vectors.

Concerning the mating behaviour of insects, it was reported that most of them mate in swarms, whereby dispersed populations aggregate at specific times and places[12,13]. In mosquitoes including malaria vectors, swarming occur very often around visual markers such as vegetation and brick piles on the ground[14–16]. This knowledge on mating places and behaviour can also help to reduce mosquito densities or interrupt the mating thus helping to reduce pathogen transmission in vector populations[17]. This technique has been effective against some Anopheles mosquitoes in Burkina Faso but on a limited scale[18]. Little information is currently available for other vector species like *An. funestus*. In *An. gambiae* s.s. mating is limited to a very short period at dusk. In this species, males always swarming before and disbanding after copulation[19]. Females approach a swarm, promptly acquire a male and leave in copula[19,20]. Mating behavior, which is one of the most important aspects of reproduction[21], remains widely under-investigated in many malaria vectors. While many studies were conducted on *An. gambiae* swarms in Western Africa[10,20,22,23], observations have rarely been reported in Eastern, Southern and Central Africa. Prior to this current report, there has been little information available on the swarms in Cameroon. Improved understanding of mosquito mating systems, and more importantly how insecticide resistance mechanisms affects the mating success in field population of malaria vectors such as *An. funestus,* could possibly give new tools for vector control implementation.

In this study, after characterizing the mating swarms of *An. funestus*, we investigated the resistance profiling and molecular basis of insecticide resistance in a natural populations of *An. funestus* in Cameroon. Furthermore, we investigated the potential impact of metabolic resistance on mating male competitiveness by assessing the association between the L119F-GSTe2 metabolic resistance marker and the mating success of *An. funestus* mosquitoes in field conditions.

Methods

Study area

Initially, the surveys covered two villages, Tibati (6°28' N, 12°37' E) and Mibellon (6°46'N, 11° 70'E) (Adamawa Region, Cameroon) but we eventually focused on just one village (Tibati) according to the density of the swarms. The main malaria vectors in Tibati are *An. funestus* during the dry season and *An. gambiae* s.l during the rainy season, whereas in Mibellon

An. funestus is the predominant species[24]. The dominance of *An. funestus* in these areas is due to the presence of multiple lakes known as suitable breeding sites for this species. LLINs is the main vector control approach in Cameroon. The villages included in this study benefited from universal LLIN distribution in 2011 and 2016. Because of high selection pressure of insecticide contained in the LLINs, the main malaria vectors have developed resistance to pyrethroids used in the nets[25]. The communities rely mainly on substance farming, agriculture but also fishing.

Detection and collection of *An. funestus* swarms

Swarm collections were undertaken on 12 evenings in February and March 2018. The search for potential swarms in each village started in the first evening at sunset around 5.30 pm, and then each swarm located were characterized and/or collected throughout the study. Swarms were searched in various places (around the potential breeding sites, closer to habitations, the farms, on the street) with the presence of potential markers assessed. For all swarms identified, different characteristics such as i) heights ii) starting time of swarming, iii) time at night when the swarms became invisible and iv) the behaviour of mosquitoes in the swarms were recorded. Swarms were then sampled using sweep nets as described previously[10,20,23]. All couples of *An. funestus* (mated) were extracted from the swarms and each couple manually transferred into a clean cartoon cup. Samples of the remaining males that did not mate in the same swarms were collected. All mosquitoes sampled were separated into unmated males, mated males and mated females and stored in RNA-later solution for further analysis.

Indoor female collections and F_1 rearing

For the purpose of assessing the susceptibility profile to various public health insecticides and WHO recommended bed nets, F_1 females were generated from indoor-resting blood-fed (F_0) females collected using electric aspirators. Collected mosquitoes were morphologically identified using the key of Gillies and De Meillon (1968). After sampling, female mosquitoes were transferred to the insectary of the Centre of Research in Infectious diseases (CRID) in Yaoundé, Cameroon. Female mosquitoes collected were kept in standard insectary conditions of 25 ± 2°C, 80 ± 10% relative humidity and fed with 10% sugar solution for at least four days and then left to oviposit using the forced-egg laying method[26]. F_1 larvae were reared to adults using the protocol previously described[26].

Species identification

Genomic DNA was extracted from 40 F_0 *An. gambiae* s.l. and 102 F_0 *An. funestus* s.l female mosquitoes (head and thorax) using the Livak protocol[27] which includes grinding of mosquito in a Livak buffer, followed by a 65°C incubation for 30 min and then centrifugation. Further steps involved an incubation on ice (30min) followed by centrifugation steps, precipitation with alcohol (100% and 70%)[27]. Mosquito species was identified using the Koekomoer cocktail Polymerase Chain Reaction (PCR) assay for *An. funestus* group and the SINE PCR assay for *An. gambiae* s.l.[28,29].

Infection of malaria vectors by *Plasmodium* parasites

Plasmodium infection rate was estimated by Taqman (401400, Santa Clara, CA, USA) assay using the head and thorax of F_0 field-collected mosquitoes as previously described[30]. 102 females *An. funestus* sensu stricto (s.s.) and 40 *An. gambiae* s.l were used for the detection of *Plasmodium falciparum* (falcip+) and/or *Plasmodium ovale*, *Plasmodium vivax*, and *Plasmodium malariae* (OVM+) sporozoites.

Insecticide susceptibility assays

Susceptibility profiles to insecticides using WHO bioassays were assessed using the F_1 generation of *An. gambiae* s.l. and *An. funestus* s.s. according to WHO procedures[31]. Insecticides tested for *An. funestus* included permethrin (0.75%) (PE 452), deltamethrin (0.05%) (DE 609), bendiocarb (0.1%) (BE 172), propoxur (0.1%), dichlorodiphenyltrichloroethane (DDT) (4%) (DD 226), malathion (5%) (MA 215), fenitrothion (1%) (FE 205) and dieldrin (4%) (DI 094) (VCRU, Penang, MALAYSIA). Due to a limited number of F_1 *An.* gambiae s.l from field collected mosquitoes, only females were tested for DDT, permethrin and deltamethrin. Control mosquitoes were exposed to non-impregnated papers. The mortality rates were determined 24h post-exposure to insecticide. In addition to the 60 min exposure described above, mortality after 30 min, 90min, 2h and 3h exposures to DDT, deltamethrin and bendiocarb was also assessed in order to evaluate the intensity of resistance of *An. funestus* s.s from Tibati.

Synergist assays

To assess the contribution of cytochrome P450 and GST enzymes in the resistance profile, synergist assays were performed with PBO (Piperonyl Butoxide) and DEM (Diethyl Maleate) with *An. funestus* s.s. Four replicates of 20–25 adult mosquitoes (2–5 day old) were immediately exposed to permethrin (0.75%), deltamethrin (0.05%), or DDT (4%) for 60 minutes after pre-exposed to PBO or DEM impregnated papers (4 %) for 1 hour. In addition, control assays using only the synergist impregnated papers for 60 minutes were also performed and mortality recorded 24 hours after. The mortality rate obtained were compared with those without synergist's exposure using a chi square test.

Assessment of bed net efficacy using cone assays

In order to assess the impact of resistance on insecticide-based interventions against the malaria vectors of this location, we checked the efficacy of common bed nets recommended by WHO against the Tibati's *An. funestus* population. 3 minute cone bioassays were carried out following the WHO guidelines[31]. Five batches of ten F_1 females (2–5 days old) were placed in plastic cones attached to 5 commercial nets: PermaNet® 2.0 (deltamethrin 1.8 g/kg) (Vestergaard, Lausanne, Switzerland), PermaNet® 3.0 (side of the net; deltamethrin 2.8g/kg) (Vestergaard, Lausanne, Switzerland), PermaNet® 3.0 (top of the net; deltamethrin 4.0 g/kg plus 25g/kg piperonyl butoxide (PBO)) (Vestergaard, Lausanne, Switzerland), Olyset® (2 % permethrin) (Sumitomo Chemical UK PLC, London, UK) and Olyset® plus (2 % permethrin plus 1 % PBO) (Sumitomo Chemical UK PLC, London, UK).

Genotyping of resistance marker and assessment of the impact on the mating male competitiveness of *An. funestus* field population

L119F-GSTe2 metabolic and A296S-RDL target-site resistance markers, involved in DDT/permethrin and dieldrin resistance in *An. funestus* respectively were genotyped in order to assess the effect of these resistance mechanisms on the mating ability of *An. funestus* field population as there is no evidence of *kdr* in *An. funestus*[32]. The L119F-GSTe2 was genotyped using an allele-specific (AS)-PCR and the A296S-RDL by TaqMan assay (Santa Clara, CA, USA). A296S-RDL TaqMan reaction was performed as previously described[33] PCR reactions (10 µl) contained 1 µl of genomic DNA, 5µl of SensiMix DNA kit (catalog: SM2-717104), 0.125µl of the A296S-RDL probe and 3.875 µl of sigma water. Samples were run on a Mx3000P™ (Stratagene) using the temperature cycling conditions of: 10 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds. We designed a new allele specific PCR to genotype the L119F-GSTe2 mutation[9]. Two pairs of primers were used for the AS-PCR (two outer and two inner primers, Table 1). Specific primers were designed manually to match the mutation and an additional mismatched nucleotide was added in the 3rd nucleotide from the 3′ end of each inner primer to enhance the specificity. PCR was carried out using 10 mM of each primer and 1ul of genomic DNA as template in 15 µl reactions containing 10X Kapa Taq buffer A (KB 1003), 0.2 mM dNTPs (DM-516404), 1.5 mM $MgCl_2$ (KB 1001), 1U Kapa Taq (KE 1000) (Kapa biosystems). The cycle parameters were: 1 cycle at 95°C for 2 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1min and then a final extension at 72°C for 10 min. PCR products were separated on 2% agarose gel by electrophoresis.

Furthermore, in an effort to characterize the broad dynamic of resistance to insecticides in this location, we also genotyped the L1014F target-site knockdown resistance (Kdr w) associated with DDT/pyrethroid resistance in *An. gambiae* using a Taqman (Santa Clara, CA, USA) method as previously described[34]. PCR reactions (10 µl) contained 1 µl of genomic DNA, 5µl of SensiMix DNA kit (catalog: SM2-717104), 0.125µl of the L1014F-kdrw probe and 3.875 µl of sigma water. Samples were run on a Mx3000P™ Multiplex quantitative PCR system (Stratagene) using the temperature cycling conditions of: 10 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds.

Statistical analysis

Association between the *GSTe2* mutation and mating success was assessed by calculating the odds ratio of mating between the homozygous resistant, heterozygous and susceptible for each gene in mated males compared to unmated group with statistical significance based on the Fisher's exact probability test. All analyses were conducted using GraphPad Prism version 7.00.

Results

Mosquito composition at Tibati

A total of 1021 blood fed female of *An. funestus* s.l. were collected indoors. Molecular identification on 102 *An. funestus* s.l mosquitoes confirmed that they were all *An. funestus s.s*. Only 40 *An. gambiae s.l* were collected and molecular identification revealed that the majority was *An. gambiae* s.s at 82.5% (33/40) whereas 17.5% (7/40) were *Anopheles coluzzii*.

Plasmodium infection rate

Out of the 102 *An. funestus* s.s tested by Taqman, 2.94% (3/102) mosquitoes were sporozoite infected with *P. falciparum.* Due to low sample size, *Plasmodium* infection rate in *An. gambiae* s.l was assessed in both species combined (*An. gambiae* (n= 33) and *An. coluzzii* (n=7)). This revealed that 12.5% (5/40) *An. gambiae* s.l. mosquitoes were infected with sporozoites predominantly with *falciparum* (falcip+; 10% [4/40]), whereas one mosquito was *P. ovale/vivax/malariae* infected (OVM+; 2.5% [1/40]). Two out of the five infected *An. gambiae s.l.* were *An. coluzzii* [2/7 infected (28.5%)] and three were *An. gambiae* [3/33 infected 9.1%)]. However, the low sample size of *An. coluzzii* means that this rate is not comparable.

Collection of the *An. funestus* swarms

15 swarms with considerably large size (more than 100 mosquitoes/swarm) were observed in Tibati, while very few swarms (with small size, less than 50 mosquitoes/swarm) were observed in Mibellon. Most mating swarms were located close to human habitations compared to other places and swarming started with two to three mosquitoes congregating after sunset around 6.05pm, and flying above a swarm place. The number of mosquitoes increased in the swarms over the next 5 to 10 minutes and slowly decreased in size then disappeared after 30 minutes when the sky became dark. Flying mosquitoes were observed by viewing them against the sky after sunset. Males of *An. funestus* swarmed at the height of 2.5m from the ground. Concerning the mating behaviour, when a female coupled with a male, they immediately left the swarms, flying at 1.5m from the ground. It is at that moment that the couples were extracted from the swarms using the sweep nets. There was no clear physical marker for *An. funestus* swarm's position in Tibati but the commonest place for swarming were just an empty space close to

Table 1. Details of primer sequences used to analyse the L119F *GSTe2* mutation.

Primers	Sequence (5' to 3')
NdeI_Gste2F	GGAATTCCATATGACCAAGCTAGTTCTGTACACGCT
XbaI_Gste2 R	TCTACATCAAGCTTTAGCATTTTCCTCCTT
L119F-Res	CGGGAATGTCCGATTTTCCGTAGAA**t**A**A**
L119-F-Sus	CATTTCTTATTCTCATTTACAGGAGCGTA**a**T**C**

habitations and most of the swarm locations remained the same for several days. Throughout this survey, we observed and collected a total of 21 copulation events in Tibati. Furthermore, we collected more than 1000 male mosquitoes from those remaining in the swarms after a mating period (that most likely did not mate). The low number of collected couples suggests a low number of females in these swarms but could also indicate that mating was also taking place in other swarms not detected in this study.

Resistance profile of malaria vectors in Tibati

An. funestus s.s exhibited full susceptibility to organophosphates (malathion and fenitrothion) and to dieldrin (organochlorine) with 100% mortality rate. This population showed high level of resistance to pyrethroids with low mortality rates in females including permethrin (type I; 26.6% ± 2.6 mortality) and deltamethrin (type II; 12.0% ± 2.3 mortality). Resistance was observed against the organochlorine DDT (46.8% ± 5.9 mortality), but only a moderate resistance was recorded against the carbamates bendiocarb (86.1% ± 5.5% mortality) and propoxur (87.2% ± 0.8 mortality) (Figure 1A). The males also exhibited similar susceptibility patterns to the females (Figure 1A). Due to the high resistance observed for pyrethroids and DDT insecticides, the intensity level of this resistance was assessed by performing bioassays with higher exposure times of 90min, 120min and 180min for deltamethrin and DDT, and also for bendiocarb (Figure 2A). After 2h and 3h exposure to deltamethrin, mosquitoes still exhibited a mortality rate of <80% (2 hours: 67.4% ± 4.5; 3 hours: 76.4% ± 3.6). In contrast, mortality rates close to 100 were observed with DDT aft 2h and 3h exposure (2h: 96.7% ± 1.7; 3 h: 100% ± 00), and for bendiocarb (90 min: 93.1% ± 1.6; 2 hours: 100% ± 00).

Analysis of *An. gambiae* s.l. mosquitoes revealed that this population was generally more resistant than *An. funestus* with lower mortality rates observed for DDT (23.6% ± 2.6 mortality), permethrin (1.75% ± 1.75) and deltamethrin (10.0% ± 5.8%) (Figure 1C).

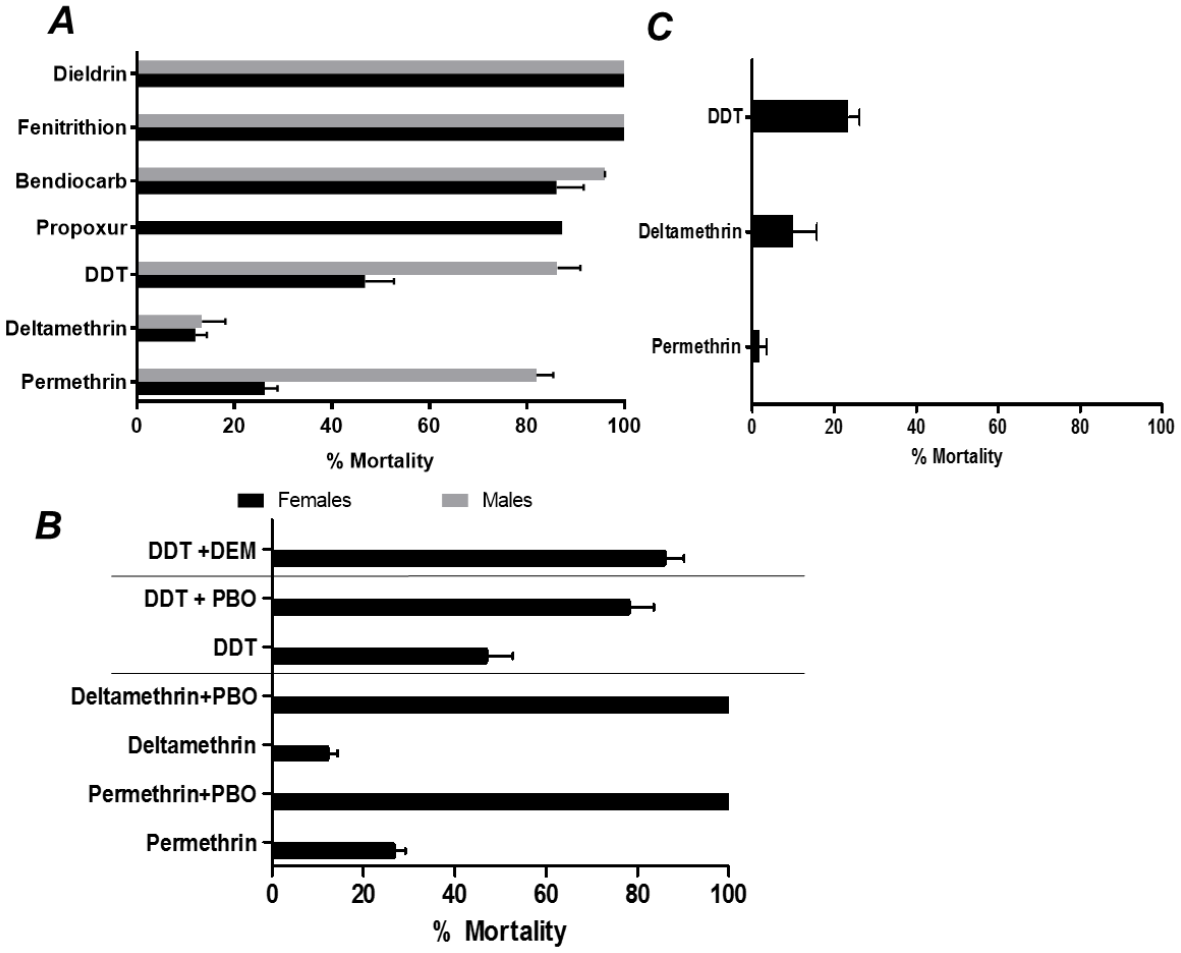

Figure 1. Susceptibility profile to main insecticides of malaria vectors in Tibati. (**A**) Susceptibility profile of *Anopheles funestus sensu stricto* and (**B**) susceptibility profile of *Anopheles funestus* s.s females after synergist assay with PBO and DEM whereas (**C**) susceptibility profile of *Anopheles gambiae* sensus lato population. Error bars represent standard error of the mean. Abbreviations: DDT, dichlorodiphenyltrichloroethane; PBO, piperonyl butoxide; DEM, diethyl maleate.

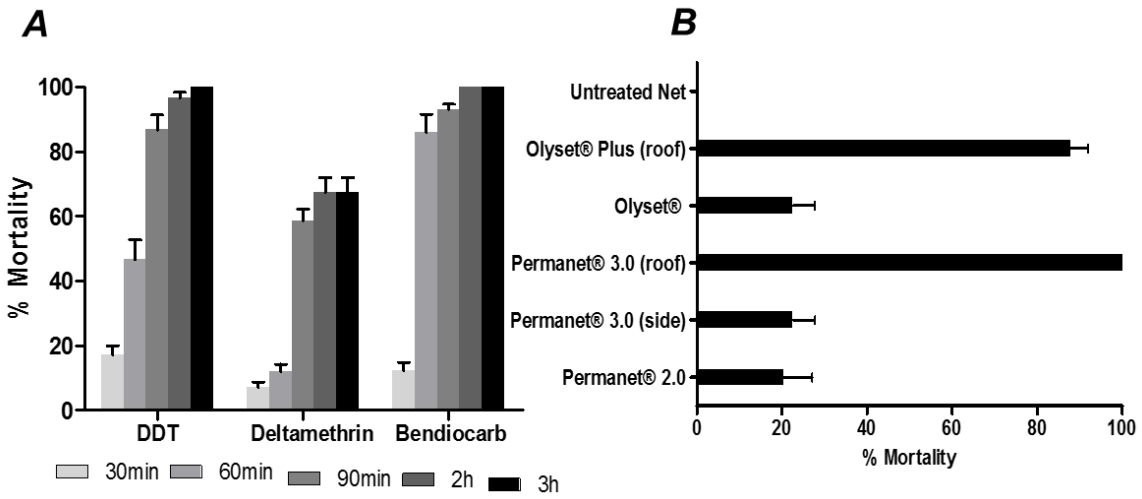

Figure 2. Exploration of resistance intensity in *An. funestus* and impact on LLINs. (**A**) Susceptibility profile at different time point exposure to DDT, deltamethrin and bendiocarb. (**B**) Bioefficacy of different commercial long-lasting insecticidal nets against *Anopheles funestus* s.s using cone assays. Error bars represent standard error of the mean.

Synergist assays

Synergist assays showed a full recovery to susceptibility after PBO pre-exposure for both type I and II pyrethroids tested (permethrin: no PBO pre-exposure (26.6% ± 2.6) mortality vs PBO pre-exposure [100.0% ± 0.0], $X^2 = 73.9$; $P < 0.0001$); deltamethrin: no PBO pre-exposure [12.0% ± 2.3%] vs PBO pre-exposure [100% ± 0.0], ($X^2 = 107.30$; $P < 0.0001$)), suggesting that cytochrome P450 enzymes may be playing a major role in pyrethroid resistance in *An. funestus* s.s. from Tibati (Figure 1B). Tests with DDT also revealed the impact of PBO pre-exposure although the susceptibility was not fully recovered (DDT: no PBO pre-exposure [46.78% ± 5.95%] vs PBO pre-exposure [78.1% ± 5.6%], $X^2 = 13.4$; $P = 0.0003$) suggesting that other gene families or mechanisms contribute to DDT resistance. For this reason, we assessed the implication of GSTs enzymes by performing a bioassay with 1h pre-exposition to DEM (inhibitors of GSTs). This revealed a recovery, although only partial (DDT: no DEM pre-exposure [46.8% ± 5.9%] vs DEM pre-exposure [85.9% ± 4.3%], ($X^2 = 22.36$; $P < 0.0001$), showing that GSTs, probably GSTe2[11], is contributing synergistically with cytochrome P450 enzymes to the resistance to DDT in this *An. funestus* population.

Bio-efficacy of commercialized nets against *An. funestus* in Tibati

A low efficacy of standard nets (Olyset and PermaNet 2.0) was observed against *An. funestus* s.s.: Olyset: 22.6 ± 5.1% mortality, PermaNet 2.0: 20.4 ± 6.7%. In contrast PBO-based nets (OlysetPlus, and PermaNet 3.0) showed an increased efficacy (OlysetPlus: 87.9 ± 3.9% mortality; PermaNet 3.0-side: 64.2 ± 6.9%, PermaNet 3.0-roof: 100.0 ± 0.0%) (Figure 2B).

Frequency of knockdown resistance (*kdr*) in *An. gambiae*

Taqman genotyping of L1014F target-site resistance mutation in *An. gambiae s.l* revealed that the frequency of 1014F kdr resistant allele was high (72.7% [48/66]) in Tibati in accordance with high pyrethroid and DDT resistance. 66.7% [22/33] were homozygote resistant, 12.1% [4/33] heterozygote whereas 21.2% [7/33] were homozygote susceptible.

Genotyping of L119F-GSTe2 metabolic resistance and impact on the mating success of *An. funestus s.s* field population

Genotyping of L119F-GSTe2 mutation in indoor collected females revealed a frequency of 28.8%, comprising 10.2% (13/127) 119F/F-RR homozygous resistant, 33.1% (42/127) 119L/F-RS heterozygotes and 56.7% (72/127) L/L119-SS homozygous susceptible (Table 2; Figure 3A). Moderate frequency of the 119F resistant allele in all samples, was recorded in mated (23.8%) compared to unmated males (33.5%) (Table 2). Direct comparison of the frequency of each genotype between mated and unmated males revealed no significant differences between all groups of mosquitoes ($P \geq 0.16$). However, an assessment of the association of each genotype with mating success using odds ratio (OR) revealed that the heterozygote genotype (L119F-RS) showed a significantly lower chance of mating than both homozygous resistant (OR = 4.2 IC: 1.49- 11.9; $P < 0.01$) and homozygous susceptible mosquitoes (OR = 7.2 IC: 3.1 - 16.8; $P < 0.0001$) (Table 3; Figure 3A). In contrast, no significant difference was observed between Homozygote resistant and homozygote susceptible mosquitoes (OR=1.77; IC 0.77-3.7; P=0.22). The impact of the resistant allele 119F on the mating competitiveness was also supported by the significantly greater likelihood of not mating when possessing this resistant allele than the susceptible L119 (OR=2.1; CI 1.1-4.0; P=0.03) (Table 4).

Genotyping of A296S-RDL target-site resistance in *An. funestus s.s*

Genotyping of A296S-RDL mutation associated with dieldrin resistance in mated and unmated males revealed that the 296S

resistant allele is almost absent in this location (Table 2, Figure 3B). These results were confirmed by the full susceptibility observed with dieldrin in the bioassay test. For this reason, no further comparison was performed for this mutation about its impact on mating success.

Discussion

Elucidating the malaria vector ecology and behaviour is crucial for the implementation of alternative control measures in order to achieve the aim of malaria elimination. Mating is one component of mosquito behaviour that remains poorly characterized. After characterizing an *An. funestus* population in Cameroon including insecticide resistance profiling and swarm patterns, we took advantage of the recent detection of the glutathione S-transferase L119F-GSTe2 marker in *An. funestus*[11] to investigate the potential influence of metabolic resistance on mating competitiveness of male *An. funestus* mosquitoes.

Table 2. Distribution of L119F-GSTe2 genotypes between mated males, mated females and unmated males compared to indoor collected females.

	Genotypes		
Phenotypes	**119F/F-RR**	**119L/F-RS**	**L/L119-SS**
Mated	4 (19%)	2 (10%)	15 (71%)
Unmated males	14 (16%)	33 (36%)	44 (48%)
Mated females	7 (33%)	4 (19%)	10 (48%)
Indoor females	13 (10%)	42 (33%)	72 (57%)
Allele	**119F**		**L119**
Mated males	23.8%		76.2%
Unmated males	33.5%	/	66.5%
Mated females	42.9%		57.1%
Indoor females	26.8%		73.2%

Species composition and their contribution to malaria transmission in Tibati

An. funestus s.s was the dominant vector in during the study coinciding with the dry season where the presence of large and permanent breeding sites as the lakes and the rivers facilitate the proliferation of this species contrary to *An. gambiae s.l*[35]. A contrasted sporozoite infection rate between both species was noticeable with high rates in *An. gambiae s.l* (12.5%), but low for *An. funestus s.s* (2.9%). The significant difference between the two species is not commonly seen in Cameroon[35,36] or DR Congo[37], as both species tend to present similar infection rates. It could be that the difference observed here is due to the ecological dynamic between the two species as it is possible that due to favorable conditions for *An. funestus*, there is an expansion of the populations of this species with more young individuals, whereas *An. gambiae* s.l population is made of older individuals in which the *Plasmodium* parasite has already completed its full extrinsic cycle since collection was done during the dry season.

High level of insecticide resistance in malaria vectors in Tibati

This study revealed a high level of resistance to multiple insecticide classes in *An. funestus s.s* and *An. gambiae s.l* which, together with their high level of *Plasmodium* infection rate, calls for urgent actions to be taken to control malaria in this region as in Cameroon. Both malaria vectors were highly resistant to pyrethroids, the only insecticide class recommended for bed nets[3]. *An. gambiae* were also found to be resistant to pyrethroids and DDT. This resistance profile is similar to that observed in Cameroon[25,38], and in Central Africa as recently reported in DR Congo[37]. Similar observations were also reported in Kenya, Madagascar, Tanzania and Uganda[39–42] where this species was highly resistant to these insecticides. The Tibati *An. funestus* population was also resistant to pyrethroids and DDT, almost at the same level as *An. gambiae*. *An. funestus* mosquitoes

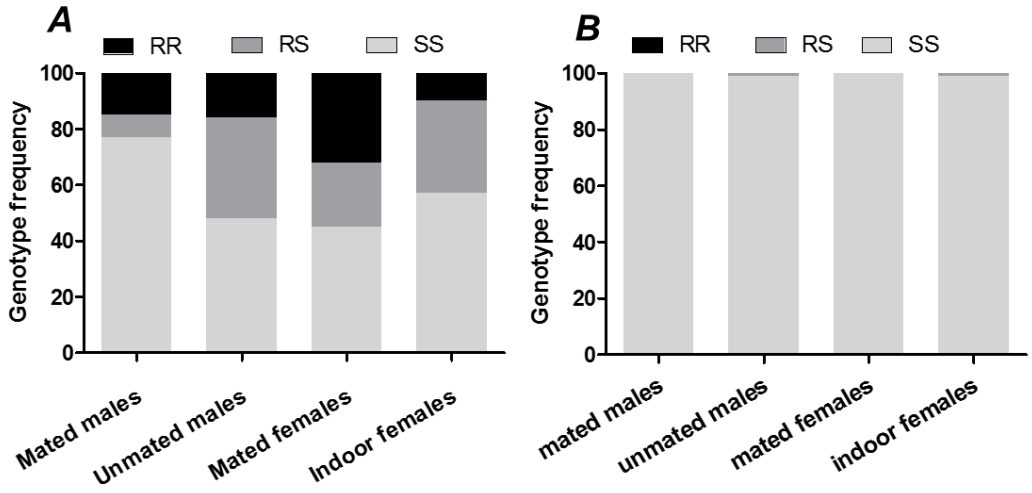

Figure 3. Distribution of resistance markers in *An. funestus* in Tibati between coupled males, uncoupled males and coupled females. (**A**) L119F-GSTe2 genotypes and (**B**) A296S-RDL genotypes.

Table 3. Distribution of A296S-RDL between mated males, mated females and unmated males compared to indoor collected females.

		Genotypes	
Phenotypes	**296S/S-RR**	**A296S -RS**	**A/A296 -SS**
Mated males	0	0	21
Unmated males	0	1	95
Mated females	0	0	17
Indoor females	0	1	126
Allele	**296S**		**A296**
Mated	0%		100%
Unmated males	0.52%	/	99.48%
Mated females	0%		100%
Indoor females	0.40%		99.60%

Table 4. Assessment of the association of different genotypes at L119F-GSTe2 mutation with mating success; *, significant difference.

Genotypes	*L119F-GSTe2*	
	Odds ratio	**P-value**
SS vs RR	**1.77** (0.77– 3.77)	0.22
SS vs RS	**7.2** (3.1 – 16.8)	<0.0001*
RR vs RS	**4.2** (1.49-11.9)	0.010*
S vs R	**2.1 (1.1-4.0)**	0.03*

showed some level of resistance to carbamates: bendiocarb and propoxur similar to reports in Northern Cameroon[43]. The common used Olyset and Permanet 2.0 LLINs presented a very low bioefficacy against *An. funestus* in cone assays. The low efficacy of this two nets, treated with permethrin and deltamethrin only, is wide-spread in *An. funestus* populations across the continent[33,37,44]. This loss of efficacy of these pyrethroid-only nets correlates well with the very high permethrin and deltamethrin resistance observed for this species. However, the greater efficacy with PBO-based nets (OlysetPlus and PermaNet 3.0) possibly provides an alternative solution to control this species for which resistance is mainly metabolic with an important role played by cytochrome P450 as shown by the synergist PBO assay. However, the spread or increased frequency of GSTe2-mediated resistance could limit the efficacy of such PBO-nets in the future. This is supported by the only partial recovery of susceptibility observed with (Olyset Plus), coupled with the increased mortality with the DEM synergist assay. The impact of GST-mediated resistance on efficacy of PBO-based nets will need to be assessed particularly in situations where such mechanism become predominant, as reported in Benin[11,45].

Swarming habits and behaviour of *An. funestus*

We observed in both Mibellon and Tibati that the heights of swarms were around 2.5m from the ground. This is in line with findings of Charlwood *et al.* in Mozambique[46], and Zawada in Zambia[47] where they noticed that *An. funestus* swarmed 2–4m from the ground. However, Harper in one study observed that *An. funestus* swarms occur immediately inside the threshold of a hut, and swarming occurred a foot off the ground[48]. Since molecular analysis were not conducted in the study of Harper, it's possible that mosquitoes he collected in the swarms was another member of the *An. funestus* group. There is also the possibility that depending on environmental conditions, *An. funestus* have changed its swarming behaviour and position. However, future studies in other locations are required to address this variation in *An. funestus* mating behaviour.

Swarming behaviour of *An. funestus* in this study was also different to that reported for *An. gambiae*. It is reported that members of the *An. gambiae* complex swarm around markers such as brick piles, rice fields, banana trees, burnt ground, garbage heaps and ant hills[49,50], however, *An. funestus* swarms we observed in this study appeared to avoid ground markers . As observed in Nchelenge, Zambia[47], there was no clear physical marker for *An. funestus* swarm's position in Tibati, but the most common place for swarming were empty spaces close to habitations, and most of the swarm locations remained the same for several days. This supports the suggestion of Charlwood *et al.* that mosquitoes of *An. gambiae* complex and *An. funestus* have different swarm markers.

As reported in other studies[12,50], mosquito swarms in Tibati occurred perpetually in the same locations at approximately the same time each day. This phenomenon needs to be assessed in other parts of Africa, which may allow the swarm to be targeted as an alternative control measure for malaria prevention. It is also unknown if *An. funestus* mate in fewer large swarms or in multiple small swarms. The number of mosquitoes in swarms as reported by Charlwood *et al.*[46] were also relatively low, and on average less than 50 adults/swarm in Mibellon. In contrast, as reported by Harper[48], about 300–500 mosquitoes were present in each swarm in Tibati during the collecting period.

Association between GSTe2-mediated metabolic resistance and mating success of *An. funestus*

This study revealed a negative impact of L119F-GSTe2 DDT/pyrethroid resistance on the mating competitiveness of males *An. funestus* as possessing the 119F resistant allele reduced the likelihood of mating. This is the first report of such negative impact of metabolic resistance on the mating success of field malaria vectors. The reduced fitness of L119F resistant mosquitoes observed in this study may suggests that the L119F mutation in the *GSTe2* gene potentially affects some physiological traits in resistant mosquitoes including mobility, perception of stimuli or even the olfactory system as the target site resistance[4,5]. However, heterozygote mosquitoes were more affected by this negative impact than homozygote resistant individuals suggesting a heterozygote disadvantage effect. In contrast, the study conducted in Vallée du Kou in Burkina Faso on the male

of *An. coluzzii* mosquitoes reported a heterozygote advantage for the target site resistance mechanisms. It was observed that *kdr* heterozygote males were more likely to mate than homozygote resistant counterparts and heterozygote RDL_R/RDL_S were also more likely to mate than homozygote-resistant males. It may be that heterozygote individuals are not affected in the same way by target site mutation and metabolic resistance driven by *GSTe2* enzymes. To confirm the lower mating ability of heterozygote mosquitoes compared to other genotypes as observed in this study, more work is needed in other locations to confirm such heterozygote disadvantage effect as the low sample size of L119F-RR homozygote resistant mosquitoes here could have impacted the assessment. Various studies conducted in other insect species on the impact of resistance on mating competitiveness showed that this trait of mosquitoes is not affected similarly. Resistant males displayed either a similar [e.g. *Metaseiulus occidentalis*:[51]], a lower [(e.g. *Anopheles gambiae*:[6])] or a higher [(e.g. *Anopheles albimanus*:[52]; *Tribolium castaneum*:[53]] mating success when compared to the susceptible counterparts. Platt *et al*. (2015) also revealed an additive mating disadvantage in male homozygotes for both kdr/RDL-resistant alleles. However, because of the low frequency of RDL it was not possible to assess the cumulative impact of target site (RDL) with metabolic (GSTe2), although this could be interesting to do in the future in populations where both types of mechanisms co-exist.

It has previously been reported that metabolic resistance mechanisms, such as the overproduction of carboxylesterases as observed in resistant *C. pipiens*, could confer a significant fitness cost on mosquitoes life-traits. It was noticed in this species that resistant individuals displayed a reduced locomotive performance compared to the susceptible ones. It was suggested that such reduced performance was caused by a resource depletion linked to the overproduction of carboxylesterases[54]. Prior to this study the only report of the impact of metabolic resistance on mating ability of malaria vector was conducted in 2015 in *An. gambiae.* Mating competitiveness in this species was not found to be significantly influenced by metabolic resistance mechanisms. However, that study[10] did not use a molecular marker for metabolic resistance, but a genome-wide microarray-based transcription analysis. The reduced performance of resistant mosquitoes in mating could contribute to slow the speed of increase in the frequency of resistant alleles in the wild, and will also prevent or delay the fixation of the resistance genes in the population. It is thus necessary that such studies are extended for other metabolic resistance mechanisms and in other locations with larger sample sizes in order to help implement successful management strategies.

Conclusion

This study revealed a high and multiple resistance to insecticides, coupled with low efficacy of LLINs without PBO in *An. Funestus,* highlighting the threat that insecticide resistance poses on the efficacy of existing vector control tools. Interestingly, this study revealed that *An. funestus* swarms can be detected and characterized in the field providing the opportunity for mating swarms of this species to be targeted to implement alternative vector control strategies. Furthermore, this study provides preliminary evidences that metabolic resistance potentially exerts a fitness cost on mating competitiveness in resistant mosquitoes. As a negative fitness costs could influence the evolution of insecticide resistance in field populations of mosquitoes like the speed of increase or reversal to susceptibility in vector populations it is crucial that such impacts are understood and taken into consideration when designing and implementing future insecticide resistance management strategies.

Data availability

Underlying data

Underlying data is available from Open Science Framework

OSF: Dataset 1. Investigation of the influence of a glutathione S-transferase metabolic resistance to pyrethroids/DDT on mating competitiveness in males Anopheles funestus, African malaria vector https://doi.org/10.17605/OSF.IO/QD8P9[55]

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I have now read the revised version of the manuscript and think that it is suitable for indexing. I have no detailed comments to give you.

Competing Interests: No competing interests were disclosed.

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

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This is an interesting manuscript that provides some comprehensive and potentially useful bionomic data for *Anopheles funestus* and, to a lesser extent, *An. gambiae* and *An. coluzzii* in Cameroon. My comments on its contents are as follows:

- The title does not adequately reflect the full scope of the content of this manuscript. Mating competitiveness is only one component of the data presented here.

- In the introduction (page 3) it is stated that little is known on the fitness costs of metabolic resistance mechanisms in anophelines owing to a lack of DNA based markers. I suggest that such markers, although of course useful, are not essential for such evaluations as fitness costs can also be assessed using biological characteristics such as life table analyses including fecundity and fertility. These likely give better indications of fitness cost. The authors should also think about what parameter they have actually measured here: was it mating competitiveness or propensity to mate? The former primarily speaks to physiological fitness while the latter is a behavioural issue. These terms may be interchangeable but there are subtle differences. Basically, less able to mate

= reduced mating competitiveness; less inclined to mate = reduced propensity.

- For the insecticide susceptibility assays, it should be noted that the standard method for assessing resistance intensity is by using increasing concentrations of the insecticide(s) in question. I assume the authors chose to use extended exposure times instead because of a shortage of test samples (F1s) but the problem with this method is that there is no clearly elucidated method for assessing the operational implications of increased intensity where detected using data generated in this way.

- The data on mating competitiveness are somewhat ambivalent because the genotype frequencies for the L119F-GSTe2 resistant homozygotes (RR) were equivalent between mated and unmated males. It is therefore difficult to see how this mutation negatively impacts mating competitiveness/propensity. As the only real difference was the frequency of heterozygotes in each group, an alternative explanation may point to an associative negative heterotic effect on propensity to mate in males that is not caused by the L119F-GSTe2 mutation itself, but rather by linkage disequilibrium between this locus and other deleterious alleles i.e. a negative pleiotropic effect.

Minor corrections:

- ‘Anopheles’ should be italicised throughout.

- Is ‘Koekemoer cocktail Polymerase Chain Reaction assay’ an official name for this assay?

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

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?
Yes

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

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

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

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

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

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mosquito diseases vector biology and control

I have read this submission. I 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.

Referee Report 29 March 2019

https://doi.org/10.21956/wellcomeopenres.16580.r35094

Adriana E. Flores
Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Mexico

The new version of the article is acceptable as it is.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: insecticide resistance in insect vectors of human disease

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

Version 1

Referee Report 20 February 2019

https://doi.org/10.21956/wellcomeopenres.16378.r34761

Adriana E. Flores
Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Mexico

This manuscript represents the evaluation of the male fitness of *Anopheles funestus* in relation with mating competitiveness with different levels of insecticide resistance to permethrin, deltamethrin and

DDT, and mechanisms involved.

The authors established the presence of CyP450 and GSTs with the use of synergists. They also determined the relationship between the L119F-GSTe2 metabolic resistance and the impact on the mating success besides the frequency of L1014F kdr mutation in the populations analyzed.

Overall the study is complete, well organized, and the analysis clearly shows the influence of the presence of the resistant allele for GSTe2 on the mating competitiveness. On the other hand, the authors found a high frequency of 1014F kdr resistance allele in the populations analyzed. I wonder, why the authors did not consider kdr resistance altogether with GSTe2. Either way, both mechanisms are present in the population. Could there be an interaction of the presence of both resistant alleles (kdr and GSTe2) in relation to the mating competition?

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

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?
Yes

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

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

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

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

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

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

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: insecticide resistance in insect vectors of human disease

I have read this submission. I 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 13 Mar 2019

Magellan TCHOUAKUI, Centre for Research in Infectious Diseases, Cameroon

We really appreciate this comment from the reviewer. We just want to highlight that a high frequency of 1014F kdr resistance allele found was for *An. gambiae* mosquitoes since for instance, there is no evidence of *Kdr* in *An. funestus*. For this reason, we could not assess the interaction of the presence of *kdr* and *GSTe2* in relation to the mating competition in *An funestus*. However, as the *RDL* and *GSTe2* are both present in this species we wanted to assess the cumulative impact of these genes on mating competition but this was not possible because of the very low frequency of the A296S-RDL mutation in this *An. funestus* population.

Competing Interests: No competing interests

Referee Report 11 February 2019

https://doi.org/10.21956/wellcomeopenres.16378.r34652

Jacques D. Charlwood
Global Health and Tropical Medicine(GHTM), Institute of Hygiene and Tropical Medicine (IHMT), NOVA University Lisbon, Lisbon, Portugal

Investigation of the influence of a glutathione S-transferase metabolic resistance to pyrethroids/DDT on mating competitiveness in males Anopheles funestus, African malaria vector.

The title of this paper is somewhat of a misnomer. It is probably described thus to attract a wider audience than a more somewhat mundane title describing the resistance status of the Anopheles funestus from a village in Cameroon. The paper describes in considerable detail the resistance status of the mosquito more than it does the effect of this on mating success. Thus, the results concerning mating success concern just 21 males caught in copula whilst the overall resistance status covers a number of tests from standard WHO tests to tests with the synergist PBO and bioassays on different net types.

The authors discuss the fact that results on mating competitiveness of resistant mosquitoes from a small number of studies give confusing results. Indeed they have participated in studies where five times the number of mating mosquitoes have been investigated. Some indicate that there may actually be an advantage whilst others give different results. It obviously is a field that can be explored further. But in the present case I feel that the authors are confusing statistical significance with biological significance. They do point out that further studies would be useful but given their title,which others may take for a fact, this is a problem.

Given that the swarms the authors observed occurred close to houses it would seem that the males rested inside houses (as they do elsewhere). It is a shame that they did not examine the proportions of the resistance genotype among these insects. Indeed, it might have been possible to collect resting insects and to examine their terminalia to determine the degree of rotation so that the effect of age on survival and resistance status among the males could have been investigated.

The authors write ‘Concerning the mating behaviour, when a female chose a male, they immediately left the swarms, flying at 1.5m from the ground.’ But it is by no means certain that female mosquitoes ‘choose’ their mates (which implies that sexual selection is taking place). With respect to their earlier paper it is perhaps worth pointing out that (as far as I know) there is no evidence of olfaction playing a part in the mating behaviour of Anopheles mosquitoes.

Given that the swarms seen were large (more than 100 individual males in a swarm) it begs the question as to how many mating pairs were seen and how many were successfully collected. If after 12 nights of observation of such large swarms only 21 pairs were formed it begs the question ‘Is this all the mating that was taking place?’ Either there were other sites where a lot more mating was taking place or something else is going on. I do not know.

The place used by the mosquitoes to swarm is similar to that described from Mozambique. There the mosquitoes actively avoid markers if they are introduced under the swarm. Again the characteristics of the location remain undefined – why they swarm where they swarm is still an enigma but as the authors point the insects remain consistent and swarm in the same place night after night.

Given the time and effort that resistance tests require their extensive data on resistance should allow them to suggest what is the optimum or minimal method that could be used to determine the resistance status in other populations. This is perhaps a better focus for their paper since much of the information that they provide is irrelevant to the title of the paper. If the paper continues with the same theme then much of the data that they present could, in fact be provided as supplemental files.

Given the advantage conferred by resistance alleles over susceptible ones and given the very high level of resistance it is strange that these genes are not fixed in the population. It also means that if in the absence of insecticide pressure resistant insects are at a disadvantage compared to the wild type (perhaps because they have to divert resources from eggs to cuticle) the presence of susceptible insects implies that once insecticide pressure is removed the population will revert back. How this might affect transmission is moot.

The English in the paper could be improved. For example, in my opinion, the very first sentence reads better thus: 'Despite an increase in the last two years1 significant progress has been made in recent decades in malaria vector control. This has contributed to a significant reduction in the burden of disease caused by this parasite'. Additionally, if the title if it is retained, can be rearranged thus ‘Investigation of the influence of a glutathione S-transferase metabolic resistance to pyrethroids/DDT on mating competitiveness in males of the African malaria vector, *Anopheles funestus*’

It is also a bit of a shame that the sporozoite data for the *Anopheles gambiae* s.l. were not given by species. With such a high rate both members of the complex would be expected to be infected. If only one species was then this would not only boost the rate – to very high levels – but would also indicate that there might be differences in vectorial capacity between the species that merit further investigation. It would seem reasonable to assume that they were dealing with ageing populations in decline after the rainy season which was responsible for the high sporozoite rates.

Presumably the A. funestus could be controlled (for the time being at least) by indoor residual spray of an insecticide like, the current flavor of the month, primiphos-methyl (Actellic).

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

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?
Yes

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

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

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

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

Are the conclusions drawn adequately supported by the results?
No

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: medical entomology

I have read this submission. I 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 13 Mar 2019

Magellan TCHOUAKUI, Centre for Research in Infectious Diseases, Cameroon

Comment 1: The title of this paper is somewhat of a misnomer. It is probably described thus to attract a wider audience than a more somewhat mundane title describing the resistance status of the *Anopheles funestus* from a village in Cameroon. The paper describes in considerable detail the resistance status of the mosquito more than it does the effect of this on mating success. Thus, the results concerning mating success concern just 21 males caught in copula whilst the overall resistance status covers a number of tests from standard WHO tests to tests with the synergist PBO and bioassays on different net types.

Response: We sincerely appreciate this comment from the reviewer. The main aim of this study was to evaluate the impact of insecticide resistance on mating competition using the L119F-GSTe2 resistance marker hence the title: ''Investigation of the influence of a glutathione S-transferase metabolic resistance to pyrethroids/DDT on mating competitiveness in males *Anopheles funestus*, African malaria vector''. Because prior to this study the resistance profile of malaria vectors in the locality was unknown, it was important to start by elucidating the resistance profile and the potential mechanisms driving the resistance to insecticide in the study site. So the title was not chosen to attract a wider audience as the reviewer is noticing but refers to the main question we wanted to address when designing this study. Also by using the word “investigation” in the title, we are acknowledging that more work is needed to fully establish this impact on mating but this provides initial useful observations on this topic.

Comment 2: The authors discuss the fact that results on mating competitiveness of resistant mosquitoes from a small number of studies give confusing results. Indeed they have participated in studies where five times the number of mating mosquitoes have been investigated. Some indicate that there may actually be an advantage whilst others give different results. It obviously is a field that can be explored further. But in the present case I feel that the authors are confusing statistical significance with biological significance. They do point out that further studies would be useful but given their title, which others may take for a fact, this is a problem.

Response: We thank again the reviewer. We agree that there is a risk of confusing statistical significance with biological significance. But we believe that we have avoided this situation by presenting each result as we saw it. We noticed when using the Odd-ratio comparison that mosquitoes with the 119F resistant allele displayed significantly lower mating competitiveness compared to those with the L119 susceptible allele and we provided the statistical significance associated with the observation. Then we discussed the potential meaning of the result but taking a cautionary approach by stating that although it could mean that there is a biological meaning that only further studies will confirm this. This is the general message of this manuscript and we agree with the reviewer that such confusion should be avoided that is why we mentioned in discussion that further studies are needed in other locations and other resistant markers to widely appreciate the impact of resistance on mating competitiveness.

Comment 3: Given that the swarms the authors observed occurred close to houses it would seem that the males rested inside houses (as they do elsewhere). It is a shame that they did not examine the proportions of the resistance genotype among these insects. Indeed, it might have been possible to collect resting insects and to examine their terminalia to determine the degree of rotation so that the effect of age on survival and resistance status among the males could have been investigated.

Response: We thank the reviewer for noting this. Our main aim as mentioned above was mainly to see if insecticide resistance has any impact on mating competition through swarm collection. That is why we did not collect resting males to examine their terminalia and determine the degree of rotation as pointed by the editor but this is a good idea that will be taken into consideration in future studies.

Comment 4: The authors write 'Concerning the mating behaviour, when a female chose a male, they immediately left the swarms, flying at 1.5m from the ground.' But it is by no means certain that female mosquitoes 'choose' their mates (which implies that sexual selection is taking place). With respect to their earlier paper it is perhaps worth pointing out that (as far as I know) there is no evidence of olfaction playing a part in the mating behaviour of Anopheles mosquitoes.

Response: We agree with the reviewer that there is very little information on the impact of olfaction on the mating behavior of Anopheles mosquitoes but in this study we noticed that when a female coupled with a male, they immediately left the swarms, flying at 1.5m from the ground for copulation although the mechanisms involved remain unknown. To avoid confusion we have replaced "chose" by "coupled with".

Comment 5: Given that the swarms seen were large (more than 100 individual males in a swarm) it begs the question as to how many mating pairs were seen and how many were successfully collected. If after 12 nights of observation of such large swarms only 21 pairs were formed it begs the question 'Is this all the mating that was taking place?' Either there were other sites where a lot more mating was taking place or something else is going on. I do not know.

Response: It is true that given the size of the swarms 21 couples collected were very low but this can be explained by the fact that females are not necessarily participating to the swarming; that is why the number of unmated males was very high. But the hypothesis that this was not all the mating taking place cannot be rejected since when the night started it was not easy to observe the couples. We have now added this sentence to highlight it: "The low number of collected couples suggests a low number of females in these swarms but could also indicate that mating was also taking place in other swarms not detected in this study."

Comment 6: The place used by the mosquitoes to swarm is similar to that described from Mozambique. There the mosquitoes actively avoid markers if they are introduced under the swarm. Again the characteristics of the location remain undefined – why they swarm where they swarm is still an enigma but as the authors point the insects remain consistent and swarm in the same place night after night.

Response: We appreciate this comment from the reviewer. Compared to *An. gambiae* complex which swarm around markers such as brick piles, rice fields, banana trees, burnt ground, garbage heaps and ant hills *An. funestus* swarms we observed in this study appeared to avoid ground markers. So it will be important in the future to assess the ecological and physiological parameters enhancing *An. funestus* mating.

Comment 7: Given the time and effort that resistance tests require their extensive data on resistance should allow them to suggest what is the optimum or minimal method that could be used to determine the resistance status in other populations. This is perhaps a better focus for their paper since much of the information that they provide is irrelevant to the title of the paper. If the

paper continues with the same theme then much of the data that they present could, in fact be provided as supplemental files.

Response: Thank you for your comments. We cannot suggest what is the optimum or minimal method that could be used to determine the resistance status in other populations since there are WHO's recommendations on how to assess the resistance profile in a given population and we were just following those instructions. We suggest keeping the whole data as it is as we explained above the insecticide work was necessary in order to explore the impact of the GSTe2 resistance allele on mating competition.

Comment 8: Given the advantage conferred by resistance alleles over susceptible ones and given the very high level of resistance it is strange that these genes are not fixed in the population. It also means that if in the absence of insecticide pressure resistant insects are at a disadvantage compared to the wild type (perhaps because they have to divert resources from eggs to cuticle) the presence of susceptible insects implies that once insecticide pressure is removed the population will revert back. How this might affect transmission is moot.

Response: Thanks to the reviewer for this remark. Some resistance genes are fixed in mosquito's populations like *Cy6p9a/b* in Southern Africa, the L119F-GSTe2 in Benin for *An. funestus* and the L1014F-Kdr in many African *An. gambiae* populations. However fixation of resistance alleles depends on other factors and the mode of selection of these alleles. That is why to it is crucial to study the impact of resistance on mosquito's life such mating competitiveness before implementing any resistance management strategy based on rotation because when the resistance becomes fixed in the population there is little chance to revert to susceptibility.

Comment 9: The English in the paper could be improved. For example, in my opinion, the very first sentence reads better thus: 'Despite an increase in the last two years1 significant progress has been made in recent decades in malaria vector control. This has contributed to a significant reduction in the burden of disease caused by this parasite'. Additionally, if the title if it is retained, can be rearranged thus 'Investigation of the influence of a glutathione S-transferase metabolic resistance to pyrethroids/DDT on mating competitiveness in males of the African malaria vector, *Anopheles funestus*'

Response: We have double-checked the whole manuscript for English grammar and mistakes.

Comment 10: It is also a bit of a shame that the sporozoite data for the *Anopheles gambiae* s.l. were not given by species. With such a high rate both members of the complex would be expected to be infected. If only one species was then this would not only boost the rate – to very high levels – but would also indicate that there might be differences in vectorial capacity between the species that merit further investigation. It would seem reasonable to assume that they were dealing with ageing populations in decline after the rainy season which was responsible for the high sporozoite rates.

Response: We combined the Plasmodium infection rate in *An. gambiae s.l* since *An. coluzzii* represented only 17.5% (*An. gambiae* (n= 33/40) and *An. coluzzii* (n=7/40)) of these mosquitoes. However, among the 5 mosquitoes infected with Plasmodium sporozoites2 were *An. coluzzii* [2/7 infected (28.5%)] and 3 were *An. gambiae* [3/33 infected 9.1%)]. However, the low sample size of *An. coluzzii* means that this rate is not comparable. Nevertheless we have now presented the infection rate for each species in the manuscript.

Comment 11: Presumably the *A. funestus* could be controlled (for the time being at least) by indoor residual spray of an insecticide like, the current flavor of the month, primiphos-methyl (Actellic).

Response: We sincerely appreciate this suggestion from the reviewer. It is true that this insecticide could be used to control this *An. funestus* population since a full susceptibility was observed for organophosphate insecticides in WHO tube assays.

Competing Interests: No competing interests