RESEARCH ARTICLE

REVISED Exploring the impact of glutathione *S*-transferase (GST)-based metabolic resistance to insecticide on vector competence of *Anopheles funestus* for *Plasmodium falciparum* [version 2; peer review: 2 approved, 1 approved with reservations]

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Abstract

Background: Malaria control heavily relies on insecticide-based interventions against mosquito vectors. However, the increasing spread of insecticide resistance is a major threat. The extent to which such resistance, notably metabolic resistance, influences the development of the *Plasmodium* parasite and its impact on overall malaria transmission remains poorly characterized. Here, we investigated whether glutathione S-transferase-based resistance could influence *Plasmodium falciparum* development in *Anopheles funestus*.
Methods: *Anopheles funestus* females were infected with *P. falciparum* gametocytes and midguts were dissected at day 7 post infection for detection/quantification of oocysts. Infection parameters were compared between individuals with different L119F-GSTe2 genotypes, and the polymorphism of the GSTe2 gene was analyzed in infected and uninfected mosquito groups.
Results: Overall, 403 *An. funestus* mosquitoes were dissected and genotyped. The frequency of the L119F-GSTe2 resistance allele was significantly higher in non-infected (55.88%) compared to infected (40.99%) mosquitoes (Fisher's exact test, P<0.0001). Prevalence of infection was significantly higher in heterozygous and homozygous susceptible genotypes (P<0.001). However, homozygous resistant and heterozygous mosquitoes exhibited significantly higher infection intensity (P<0.01). No association was observed between the GSTe2 polymorphism and the infection status of mosquitoes.
Conclusion: Altogether, these results suggest that GSTe2-based metabolic resistance may affect the vectorial competence of resistant *An.*

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funestus mosquitoes to *P. falciparum* infection, by possibly increasing its permissiveness to *Plasmodium* infection.

Keywords
Malaria, Insecticide resistance, Anopheles funestus, Plasmodium falciparum, metabolic resistance, GSTe2

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

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REVISED **Amendments from Version 1**

We express our sincere thanks and gratitude to the editor and to the reviewers for their constructive comments and for the time they have taken to revise our work in such a thoughtful way. We have taken into consideration the comments and suggestions made by the editor and the reviewers and attempted to integrate them in the best possible way. All typos and language errors have been corrected according to reviewer's comments/suggestions. Sentences which were long (especially in the discussion) have been shortened and others have been reformulated or modified, according to reviewer's recommendations, to make more sense.

The introduction's last paragraph has been rewritten. In the methods section, we provided more details about study sites including species of *An. gambiae* complex which are present and history of insecticide resistance. Talking about the number of sequences analyzed we argued that although analyzing 30 sequences out of individuals out of 400 seems small, it is sufficient to provide appropriate tendencies, by showing that number of studies published in peer reviewed journals have generated very robust results by analyzing similar sample size or less per population.

Moreover, this preliminary study on the impact of GSTe2-based metabolic resistance on *An. funestus* vector competence showed an interesting trend that metabolic resistance possibly impacts vector competence by increasing parasite load. Our findings are consistent with what has been reported on the field, where GSTe2 resistant genotypes were significantly more infected by *P. falciparum*. Nevertheless, we agreed with the reviewers and mentioned in the discussion that our study should be followed by further studies using mosquitoes from the same locality that will be fed using the same infectious blood to avoid confounding factor due to difference in gametocytemia. This will help to have more comprehensive understanding of the impact of metabolic resistance on *An. funestus* vector competence.

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

Introduction

Intense control efforts have been deployed since 2000 to reduce the burden of malaria in Africa, relying heavily on insecticide-based interventions, including the scale-up of long-lasting insecticide nets (LLINs) and indoor residual spraying (IRS). The proportion of households possessing at least one LLIN has increased from less than 50% in 2010 to an estimated 80% in 2016. Similarly, the proportion of populations at risk of malaria sleeping under LLINs has increased from 24% to 54% in the same time frame (WHO, 2017). The implementation of these vector control measures led to significant reduction of malaria incidence and mortality by 21% and 31%, respectively on the African continent, between 2011 and 2015 (WHO, 2017). Unfortunately, the heavy use of insecticides in the public health and agriculture sectors has in turn selected resistance in major vector mosquitoes *An. gambiae*, *An. coluzzii* and *An. funestus s.s.* (hereinafter *An. funestus*) across the continent, and this is considered as a serious threat to sustainable malaria control (Ndo *et al*., 2018; Ranson & Lissenden, 2016). There is a fear that such resistance could impact malaria vector competence and increase malaria transmission. However, little is known on the interactions between resistance and mosquito's ability to harbour and transmit malaria parasites, preventing us from anticipating the epidemiological impact of insecticide resistance.

In *Anopheles* mosquitoes, insecticide resistance is driven mainly by two mechanisms: alteration of target sites of insecticides and metabolic resistance through an over-expression of detoxification genes (Corbel *et al*., 2007; Liu, 2015; Menze *et al*., 2016). The target-site insensitivity resistance is the best characterized and can be easily monitored using various diagnostics (Bass *et al*., 2007; Martinez-Torres *et al*., 1998). Point mutations in the gene coding for the voltage-gated sodium channel confer cross resistance to dichlorodiphenyltrichloroethane (DDT) and pyrethroids insecticides named for knockdown resistance (kdr) (Ranson *et al*., 2000), while mutations in the *ace-1* gene, which encodes the acetylcholinesterase enzyme, confer cross-resistance to carbamate and organophosphate insecticides (Alout & Weill, 2008; Weill *et al*., 2004). Contrary to the target site mechanism, monitoring of metabolic resistance is more complex and requires advanced genomic analytical methods such as qPCR, microarrays or RNA sequencing. Metabolic mechanisms are the result of over-expression, either by amplification and/or upregulation of detoxification genes (Cytochrome P450s, glutathione S-transferases and esterases) (Hemingway & Ranson, 2000).

The successful management of insecticide resistance will require a good understanding of the mechanisms involved, but more importantly, its impact on vectorial capacity/competence and malaria transmission. The selection of insecticide resistance in mosquitoes is thought to interfere with the pathogens they transmit during one of the main steps of development, including parasite differentiation, proliferation, and migration to the specialised tissues. For example, enzymatic modifications involved in metabolic resistance could render mosquito internal environment toxic for the parasite or may influence one or many steps of the immune response, from the recognition of the parasite as foreign, to the deployment of the killing mechanism (Rivero *et al*., 2010).

Despite the widespread distribution of resistance, its impact on the ability of *Anopheles* vectors to transmit malaria and therefore, its epidemiological impact remains unclear. This is particularly true for metabolic resistance mechanisms since no molecular marker was previously available to assess its impact contrary to target-site resistance [e.g. knockdown resistance (*kdr*)] for which DNA-based diagnostic tools were designed two decades ago (Martinez-Torres *et al*., 1998). Progress made recently in *An. funestus* has led to the detection of a DNA-based marker for the glutathione S-transferase epsilon 2 gene (*GSTe2*) consisting of one single amino acid change (L119F) in an upregulated *GSTe2* (Riveron *et al*., 2014). Geographical distribution of this point mutation strongly correlated with insecticide resistance patterns across Africa. Functional characterization of recombinant *GSTe2* further supported the resistant allele as being more efficient at metabolizing insecticide, notably DDT, by enlarging the GSTe2-DDT biding cavity, leading to increased access and metabolism of the insecticide (Riveron *et al*., 2014). Taking advantage of availability of this new DNA-based *GSTe2* marker, we investigated the impact of a GST-mediated metabolic resistance on the vector competence of the major malaria vector *An. funestus*. We showed that the L119F-GSTe2 mutation conferring pyrethroid/DDT resistance could influence *P. falciparum* infection in field populations of this vector.

Methods

Study sites

Mosquitoes originated from Mibellon (6 ° 46'N, 11 ° 70'E) and Obout (3° 7'N, 11 ° 65'N) situated 350 km and 25 km, respectively, from Yaoundé the capital city of Cameroon. Mibellon is situated in the Adamaoua region in the humid savannah zone. The climate is Sudano-Guinean characterized by an eight-months rainy season from March to October, and a dry season of four months extending from November to February (Olivry, 1986). Two main malaria vector species namely *An. gambiae sl* and *An. funestus* are routinely found in the village, with the latter being the most abundant throughout the year. Both vectors have developed high levels of resistance to pyrethroids (deltamethrin and permethrin) and organochlorides (DDT) and moderate resistance to carbamate (bendiocarb) (Menze *et al.*, 2018). *Anopheles funestus* was found to actively transmit *Plasmodium* parasite in the locality, with an infection rate of up to 3.7% (Menze *et al.*, 2018; Ndo *et al*., 2016).

Obout is located within the dense rainforest area of the Centre region (Southern Cameroon). The climate is similar to that of Equatorial Guinea, characterized by two rainy seasons extending from August to October, and from April to June. There are also two dry seasons running from November to April, and from June to July (Olivry, 1986). *Anopheles gambiae sl* and *An. funestus* are the main vector species found in the village (Ndo *et al.*, 2018). High *Plasmodium* infection rates reaching 23% were reported in these species which have also developed resistance to DDT and pyrethroids, notably deltamethrin and permethrin (Ndo *et al*., 2018).

Mosquito sampling and identification

Mosquitoes were collected between 8–11am inside human dwellings using electric aspirators (Rule In-Line Blowers, Model 240). They were brought back to the insectary where initial species identification was performed based on morphological criteria (Gillies & Coetzee, 1987). They were later confirmed as *An. funestus* using a PCR assay (Koekemoer *et al.*, 2002). All blood-engorged *An. funestus* mosquitoes were kept four days in cages until eggs were mature. Gravid females were allowed to oviposit individually using a forced egg-laying method (Morgan *et al.*, 2010). Progenies were pooled and reared to adulthood under standardised conditions.

Experimental infections

A total of 20 infection experiments were conducted, each using blood from different gametocyte carriers, with different parasite density. Gametocytes of *P. falciparum* were collected from the blood of infected children at local primary schools of the locality of Okola (Centre, Cameroon) as previously described (Ndo *et al.*, 2016). Briefly, presence of different parasite stages in the blood was detected by examining thick blood smears stained with 10% Giemsa under light microscope (Leica DM 300). The number of gametocytes was counted against 500 leucocytes, and an estimation of its density in the blood was done based on an average of 8000 white blood cells /µl.

Blood was collected from selected gametocyte carriers by venepuncture into heparinized tubes. It was immediately centrifuged for 5 minutes at 2000 RPM using a centrifuge (Model EBA 20, Hettich Lab Technology) placed inside an incubator (Jouan EB115) set at 37°C, and the donor's plasma was replaced by the same volume of European AB malaria-naïve plasma (Catalogue number H4522, Sigma-Aldrich, Taufkirchen, Germany). Three to five day old F1 female mosquitoes were allowed to feed through an artificial parafilm membrane maintained at 37°C using a circulating heating water bath (Fisher Scientific INC, Isotemp 4500H5P, Pittsburgh USA). After 45 min, fed mosquitoes were sorted and placed in separated cups until dissection of midguts at day 7 (D7) post-infection. Dissected midguts were stained with 0.4% mercurochrome before they were examined under light microscopy (Leica, Model DM 300) at objective 40X for detection and quantification of oocysts. All carcasses were preserved at -20°C until DNA extraction was performed.

Throughout the experiments, we used the *An. coluzzii* Ngousso strain as control sample to monitor for the effectiveness of blood handling procedure and infectivity of gametocytes, since this strain is well adapted to feed on artificial parafilm membrane and is known to be highly susceptible to *P. falciparum* infection (Ndo *et al.*, 2016). The *An. coluzzii* Ngousso strain originated from Yaoundé (Cameroon) and is routinely maintained at the insectary of the Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale (OCEAC, Cameroon) since 2006.

L119F-GSTe2 mutation genotyping

Genomic DNA was extracted using Livak's method (Livak, 1984) from carcasses of infected and non-infected mosquitoes after dissection of midguts. Genotypes of L119F-GSTe2 mutation were determined after DNA amplification using allele specific PCR diagnostic assays using two outer and two inner primers (Tchouakui *et al.*, 2018). Details of the sequences of primer used are presented in Table 1. PCR was performed in Gene Touch

Table 1. Details of primer sequences used to genotype *L119F GSTe2* mutation in *Anopheles funestus*.

Primers	Sequence (5' to 3')
Ndel_Gste2F	GGAATTCCATATGACCAAGCTAGTTCTGTACACGCT
Xbal_Gste2 R	TCTACATCAAGCTTTAGCATTTTCCTCCTT
L119F-Res	CGGGAATGTCCGATTTTCCGTAGAA**t**A**A**
L119-F-Sus	CATTTCTTATTCTCATTTACAGGAGCGTA**a**T**C**

thermalcycler (Model TC-E-48DA), in a reaction volume of 15 µl using 10 µM of each primer, 10X Kapa Taq buffer A, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1U Kapa Taq (Kapa biosystems 5U/µl, Cat: 07958471001) and 1µl of genomic DNA as template. The initial denaturation step at 95°C for 2 min was followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final extension step at 72°C for 10 min. PCR products were separated on 2% agarose gel allowing clear discrimination of the genotypes. The size of the diagnostic band was 523 bp for homozygous resistant (RR) and 312 bp for homozygous susceptible (RS), while heterozygous (SS) showed the two bands.

GSTe2 gene sequencing

A total of 30 DNA samples including 15 of infected and 15 of non-infected mosquitoes were randomly selected for sequencing of the *GSTe2* gene using the following primers: Gste2F, 5'GGA ATT CCA TAT GAC CAA GCT AGT TCT GTA CAC GCT 3' and Gste2R, 5' TCT AGA TCA AGC TTT AGC ATT TTC CTC CTT 3' (Eurogentec, Liège Science Park, Belgium). DNA was amplified in a total volume of 15 µl containing 10 µM of each primer (forward and reverse), 10 mM dNTPs, ddH_2O, 10X buffer A, 1.5 mM $MgCl_2$ and 1ul of Kapa Taq polymerase (KapaBiosystems, Cat: 07958471001). PCR conditions were an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, with final extension at 72°C for 10 min. The size of amplicons was checked after visualization of DNA bands on a 2% agarose gel stained with GelRed nucleic acid dye (Biotium, Cat: 41003) (see Underlying data (Ndo, 2019)). PCR products were purified using ExoSap PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA; Cat:78201.1.ML) following manufacturer's instructions, and was sequenced using the forward and reverse primers.

Data analysis

Parameters analyzed for each infection experiment were the prevalence of infection, as the proportion of mosquitoes infected after midgut dissection, and the infection intensity by calculation of arithmetic mean and median number of oocysts in the midguts of infected mosquitoes.

The geographical distribution of L119F-GSTe2 mutation was assessed by determining allelic and genotypic frequencies in each study sites. The impact of L119F-GSTe2 mutation on vector competence was investigated by comparing the frequency of the L119F-GSTe2 resistant allele in infected and non-infected mosquitoes, and by comparing infection parameters (Prevalence of infection, mean, median oocyst load) between mosquitoes of different genotypes (RR, RS and SS). Prevalence of infection, mean, and median oocyst load were computed and compared using the Fisher's exact test and Mann-Withney test, respectively. P-values less than 0.05 were considered as statistically significant. The software GraphPad Prism v 7.05 was used for all statistical analysis.

Genomic sequence analysis started with systematic detection and correction of base-calling and/or sequencing errors using Bioedit V.7.2.5, after visual inspection of DNA sequence chromatograms. A consensus sequence for each single mosquito was generated using both forward and reverse sequences which were used for analysis of polymorphisms and phylogenetics. Sequences were aligned using MEGA V.6.06 and DNA polymorphism parameters were generated in dnaSP V.5.10. Haplotype networks and maximum likelihood phylogenetic tree were constructed using TCS V.1.21.

Ethical statements

The study was approved by the Cameroonian Ethical Committee for Research in Human Health (Statement N°2016/10/817/CE/CNERSH). The gametocyte carriers used in this study were enrolled as volunteers. Their parents or legal guardians signed a written informed consent form after the procedures of the study were fully explained to them. All children found infected with the malaria parasites received free antimalarial treatment.

Results

Mosquito species identification

In Obout, 615 *Anopheles* mosquitoes were collected during the study period. According to morphological identification, 91.38% belonged to the *An. funestus* group while the remaining mosquitoes were all *An. gambiae sl* (8.62%). In Mibellon, *An. funestus* was also the main vector species representing 94.92% of the 670 *Anopheles* mosquitoes collected while *An. gambiae sl* represented the rest (5.08%). The molecular species identification of the *An. funestus* group showed presence of *An. funestus s.s.* (97.38%) and *An. leesoni* (2.62%) in Obout, while only *An. funestus s.s.* (hereafter called *An. funestus*) was present in Mibellon.

Anopheles funestus infection

Overall, the blood feeding rate of *An. funestus* through the artificial parafilm membrane was low compared to that of the *An. coluzzii* Ngousso strain used as control (80.42% - 100%) and did not exceed 40% in all the cases (min - max: 1% - 38%). *Anopheles funestus* of both sites showed high susceptibility to natural *P. falciparum* isolates with 72.73% (8/11) and 77.78% (7/9) experiments yielding at least one infected mosquito in Obout and Mibellon, respectively. The overall infection rate was 69.73% Mibellon and 42.74% in Obout. By contrast infection intensity represented by mean and median oocyst load in midgut was moderate in Obout (mean: 7.44±1.20; median: 4) and low in Mibellon (mean: 2.88±0.18; median: 2). (Table 2).

Distribution of L119F-GSTe2 resistance allele and *P. falciparum* infection

A total of 218 and 185 mosquitoes was genotypes in Obout and Mibellon respectively. A non-uniform geographical distribution of L119F-GSTe2 resistance allele was observed with a higher frequency in Obout (65.93%) compared to Mibellon (25.95%). Distribution of *GSTe2* genotypes showed that homozygous resistant (RR: 50.40%) and heterozygous (RS:31.04%) were the most frequent in Obout, while SS (60%) was predominant in Mibellon (Figure 1). For analysis of impact of *GSTe2* resistant allele on *An. funestus* vector competence, only experiments for which at least 20% of prevalence of infection was observed were

Table 2. **Infection parameters in *An. funestus* from Obout and Mibellon.**

Sites	Exp	Dissected	Infected	Infection rate (%)	Total oocyst	Mean oocyst	Median oocyst	Oocyst range
Obout	N°1	58	38	65.52	177	4.66±0.55	4	1 – 16
	N°2	61	17	27.87	39	2.29±0.41	2	1 – 8
	N°3	22	5	22.73	13	2.6±1.03	1	1– 6
	N°4	27	22	81.48	449	20.41±4.71	13.5	1 – 92
	N°5	22	10	45.45	64	6.4±1.94	6	1 – 21
	N°6	28	12	42.86	45	3.75±0.74	3	1 – 8
	N°7	24	1	4.17	1	1	1	1
	N°8	6	1	16.67	1	1	1	1
	ALL	248	104	41.93	787	7.57±1.22	4	1–92
Mibellon	N°9	9	4	44.44	13	3.25±0.63	3	2–5
	N°10	31	17	54.84	27	1.59±0.21	1	1–4
	N°11	14	5	42.86	12	2.4±0.4	3	1–3
	N°12	53	32	60.38	73	2.28±0.20	2	1–6
	N°13	7	7	100	31	4.43±0.89	4	1–8
	N°14	58	52	89.65	181	3.48±0.34	3	1–12
	N°15	13	12	100	35	2.92±0.62	2	1–8
	ALL	185	129	69.73	372	2.88±0.18	2	1–12

Gametocyte density is expressed as number of gametocytes per µl of blood assuming an average of 8000 white cells/µl.

Exp: experiment; N°: number.

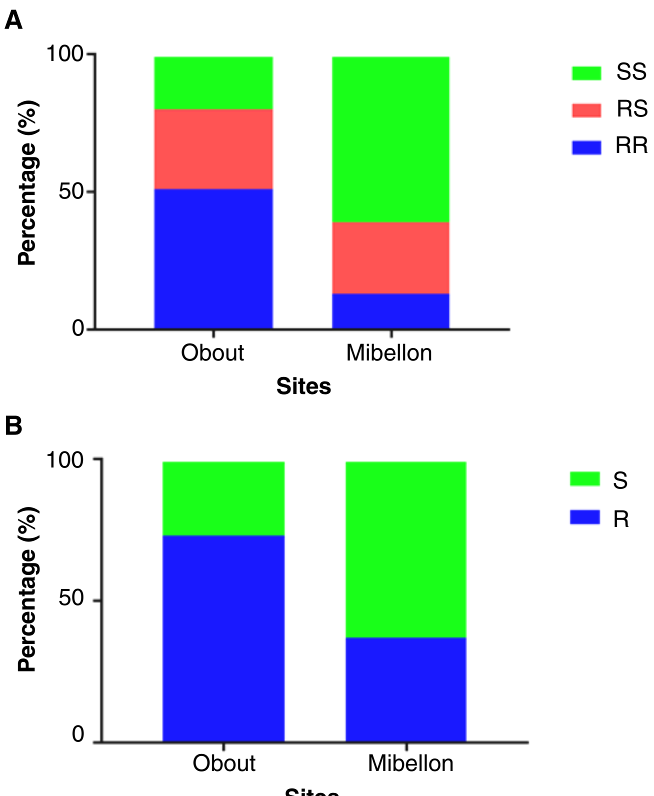

Figure 1. **Distribution of L119F-GSTe2 resistance genotypes (A) and alleles (B) in Obout (N:128) and Mibellon (N:185). (SS: homozygous susceptible genotype, RS: heterozygous genotype, RR: homozygous resistant genotype, R: resistant allele, S: susceptible allele).**

considered (Table 2). The frequency of the L119F-GSTe2 resistance allele was significantly higher in non-infected (55.88%) compared to infected (40.99%) mosquitoes (Fisher's exact test, P<0.0001). Heterozygous (RS:58.14%) and susceptible (SS:64.33%) mosquitoes were significantly more infected than their homozygous resistant (RR:40.14%) counterparts (Fisher's exact test, P: 0.0037 for RR vs SS; P<0.001 for RR vs SS; P:0329 for RS vs SS) (Figure 2). As such, the odds ratio of being infected were significantly higher in RS and SS than in RR (OR: 2.07; 95%CI: 1.28-3.35 for RS vs RR; OR: 2.69, 95%CI:1.69-4.28 for SS vs RR; OR: 0.77, 95%CI: 0.48-1.24 for RS vs SS). The results of infection intensity were conflicting since mosquitoes bearing the resistant allele appeared to be much more permissive to oocyst infection (Figure 3). Overall the number of oocyst found in a single midgut was significantly higher in heterozygous (Mean ± SEM: 5.80±0.77; Median: 3) and homozygous resistant genotypes (Mean ± SEM: 7.30±1.94; Median: 3) compared to susceptible mosquitoes (Mean ± SEM: 2.92±0.26; Median: 2) (Mann-Withney test, P:0.0323 for RR vs SS; P:0.0007 for RS vs SS; P:0.5011 for RR vs RS).

GSTe2 gene polymorphism and *Plasmodium* infection

A total of 34 sequences belonging to infected (N=18) and non-infected (N=16) mosquitoes were analyzed. The fragment length was 787 bp, spanning 3 exons and 2 introns and covering 92.6% of the full *An. funestus GSTe2* sequence (AFUN015809-RA). Performing a BLASTn search in Vectorbase using the *An. funestus* sequence generated in this study revealed a very high-sequence homology (99.4%) with *An. funestus* full *GSTe2* gene sequence.

Overall the diversity of the *GSTe2* fragment analyzed was low with only 15 (1.91%) polymorphic sites. All the 15 polymorphic sites were present in the non-infected group, while only 11 were found in infected group. This means that, nucleotide diversity was a bit higher in non-infected (π=0.005) compared to the infected (π=0.003) group. The mean numbers of nucleotide differences of the non-infected and infected groups were 3.667 and 2.510, respectively (Table 3). However, no fixed mutation was observed between sequences of infected and non-infected mosquitoes.

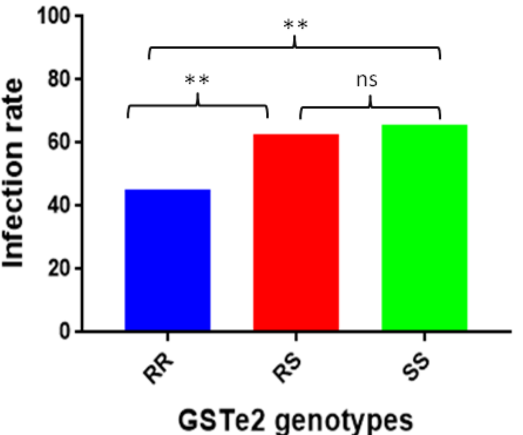

Figure 2. Prevalence of infection according to L119F-GSTe2 genotypes. **: P<0.001; ns: not significant. (SS: homozygous susceptible genotype, RS: heterozygous genotype, RR: homozygous resistant genotype).

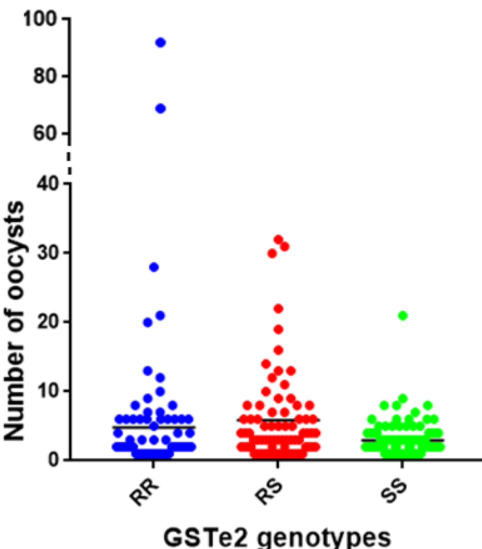

Figure 3. Infection intensity according to L119F-GSTe2 genotypes. Each dot represents a number of oocysts in a single midgut. It is possible that some dots are superposed. (SS: homozygous susceptible genotype, RS: heterozygous genotype, RR: homozygous resistant genotype).

The substitutions defined 14 nucleotide haplotypes. Sequences of haplotypes have been deposited in GenBank under accession numbers MK439920 - MK439933. Haplotype diversity was slightly higher in the non-infected (0.883) compared to the infected group (0.797) (Table 2). Six (42.86%) haplotypes appeared among non-infected specimens (Hap_9 - Hap_14), while 5 (35.71%) haplotypes were present in infected samples (Hap_2 - Hap_3, Hap_6 - Hap_8). Only 3 (21.43%) of the 14 haplotypes were shared between the two groups (Figure 4). The major haplotype grouped 13 of 34 sequences (9 of RR and 4 of RS genotypes) and was shared between non-infected (61.54%) and infected (38.46%) samples. The haplotype network and the maximum likelihood phylogenetic tree did not reveal a clear segregation of haplotypes or individuals of these groups (Figure 4). Moreover, Tajima's D, although negative were statistically non-significant (P>0.05) in all groups, suggesting no evidence of signature of selection (Table 3).

Discussion

In this study the distribution of the L119F-GSTe2 resistance allele and its impact on *P. falciparum* infection were assessed using mosquitoes originating from Obout and Mibellon (Cameroon). In these sites, *An. funestus* was by far the most abundant species collected, probably breeding in swamps formed by numerous rivers and lakes that promote the practice of fishing. This observation is in line with results of previous studies conducted in the same localities, during which high densities of

Table 3. **Summary of *GSTe2* sequence polymorphisms in infected and non infected mosquitoes.**

		Conding region				Non-coding region			Whole sequence
		Exon1	Exon2	Exon3	All	Intron1	Intron2	All	
Infected	N seq	18	18	18	18	18	18	18	18
	N indiv	9	9	9	9	9	9	9	9
	Size	107	202	309	643	72	72	144	787
	Poly sites	2	1	2	6	4	1	5	11
	H	3	2	3	7	5	2	6	8
	Hd	0.307	0.209	0.529	0.791	0.484	0.336	0.562	0.797
	pi	0.003	0.001	0.002	0.002	0.009	0.005	0.007	0.003
	K	0.320	0.209	0.765	1.503	0.641	0.366	1.007	2.510
	Fu Li D	-0.552	0.667	0.885	0.577	-0.070	0.667	-0.359	0.164
	Fu Li F	-0.798	0.405	0.977	0.336	-1.008	0.708	-0.607	-0.121
	Tajima's D	-1.096	-0.529	0.769	-0.331	-1.347	0.488	-0.974	-0.786
Non infected	N seq	16	16	16	16	16	16	16	16
	N indiv	8	8	8	8	8	8	8	8
	Size	107	202	309	643	72	72	144	787
	Poly sites	1	2	3	6	3	6	9	15
	H	2	2	4	6	4	4	6	9
	Hd	0.325	0.125	0.692	0.842	0.517	0.442	0.675	0.883
	pi	0.003	0.001	0.003	0.003	0.008	0.021	0.014	0.005
	K	0.325	0.250	1.058	1.633	0.575	1.458	2.033	3.667
	Fu Li D	0.688	-1.915	-0.039	-0.706	-1.122	0.612	-0.051	-0.369
	Fu Li F	0.627	-2.060	0.117	-0.694	-1.262	0.306	-0.333	0.544
	Tajima's D	0.156	-1.498	0.495	-0.331	-1.055	-0.662	-0.919	-0.740

N seq: number of sequences; N indiv: number of individuals; Poly sites: polymorphic sites; H: haplotypes; Hd: haplotype diversity; Pi: nucleotide diversity; K: average number of nucleotide differences.

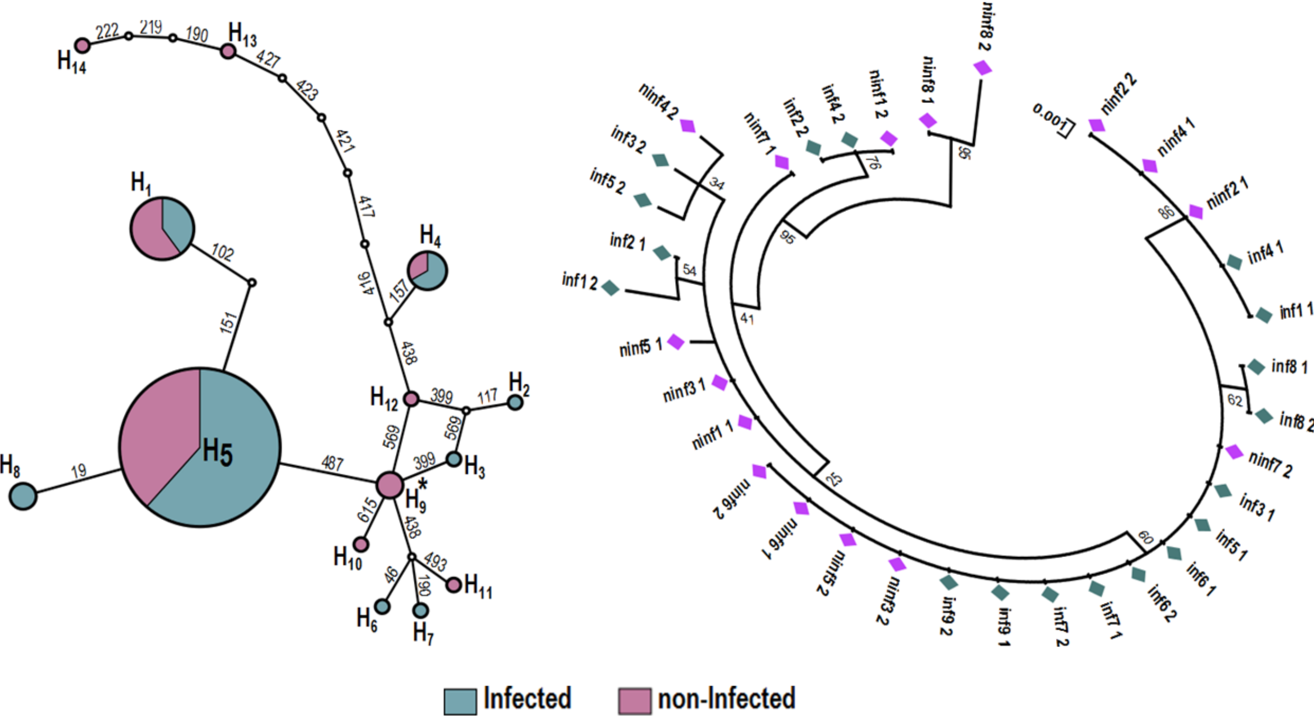

Figure 4. **Haplotype network (left) and maximum likelihood phylogenetic tree (right) of *GSTe2* haplotypes in infected and non-infected *Anopheles funestus* mosquitoes.**

resting blood fed *An. funestus* were collected in human dwellings. Using the Taqman technique, elevated *P. falciparum* infection rates reaching 23% were detected in this vector, thus underlying the major role it plays in malaria transmission (Menze *et al*., 2018; Ndo *et al*., 2018; Ndo *et al*., 2016). All together these findings indicate that in these localities *An. funestus* remains an important malaria vector, despite large coverage of insecticide treated nets. It is possible that the high level of insecticide resistance detected in vector populations of the localities surveyed is one of the key factors responsible for the reduced efficacy of these vector control tools (Menze *et al*., 2018; Ndo *et al*., 2018; Ndo *et al*., 2016).

A non-uniform geographical distribution of L119F-GSTe2 resistance allele was observed in the two *An. funestus* populations. The frequency of the allele was very high in Obout (65.93%) while it was low in Mibellon (29.95%). This result, particularly in Mibellon, did not correlate well with the high resistance to permethrin (mortality 48.88 ± 5.76%), deltamethrin (mortality was 38.34 ± 5.79%) and DDT (55.28 ± 8.28%) previously reported in this site, indicating that other mechanisms, such as overexpression of cytochrome P450s, could be involved (Menze *et al*., 2018). Furthermore, this non-uniform distribution of the L119F-GSTe2 resistance allele also suggests that restriction of gene flow may exist between *An. funestus* populations in Cameroon, and/or that different selection pressures could have selected the resistance to insecticides in this species in both localities.

Whether the L119F-GSTe2 resistance allele impacts vector competence of *An. funestus* was investigated by comparing infection prevalence and intensity between mosquitoes belonging to RR, RS and SS genotypes, and by analyzing the polymorphisms in the *GSTe2* gene in infected and uninfected mosquitoes. We used the parafilm-glass feeding system for experimental infection of *An. funestus*. This technique can now be routinely used in this mosquito, similarly to *An. gambiae sl*, despite the observation that blood feeding rates remain low, likely because freshly field collected mosquitoes are not well adapted to feed through an artificial membrane (Ndo *et al*., 2016). Similarly to the study of Ndo *et al.* (Ndo *et al*., 2016), the two *An. funestus* populations used in this study also showed high susceptibility to natural *P. falciparum* gametocyte, a result that further confirms that this species is highly susceptible to the malaria parasite, hence its major role in the transmission of this disease in sub-Saharan African settings. By detecting higher overall infection rate in Mibellon, but higher infection intensity in Obout, our results did not allow us to clearly establish whether *An. funestus* populations from rainforests (Obout) are more or less competent to develop/transmit the malaria parasite than those from the humid savannah (Mibellon). Further studies using several mosquito populations from both ecoclimatic zones and fed on the same infectious blood are necessary. Sequence analysis did not reveal an association between *GSTe2* polymorphism and infection in field *An. funestus* mosquitoes, although sequencing more samples in the future could help to confirm this. On the other hand, infection prevalence and intensity were compared between mosquitoes of different genotypes, at oocyst level. It could have been also interesting to examine this impact at sporozoite level, which is the parasite stage transmitted to humans. However, the cumulative effect of the low number of mosquitoes fed on artificial membrane, the number dissected at day 7 and mosquito mortality before day 14, when sporozoites can be found in salivary glands, prevented us from carrying out this analysis. Nonetheless, analyzing such impact at oocyst level is reliable, as it has been shown that mosquito infectivity can be predicted with reasonable certainty from oocyst prevalence including in the cases of low intensity infections (Stone *et al*., 2013). In general, the results obtained were contrasting. Both insecticide susceptible homozygote (SS) and heterozygote (RS) genotypes were significantly more susceptible to *P. falciparum* infection than the homozygote resistant (RR) genotype. In contrast results on the intensity of infection show that mosquitoes bearing the resistant L119F-GSTe2 allele (RR and RS genotypes) are more permissive to parasite infection than those with the SS genotype. This later observation suggest that GST-based metabolic resistance might interact with the immune system of the mosquito leading to a significant development of the parasite (Ndiath *et al*., 2014; Rivero *et al*., 2010). Here, we suggest two possible explanations. The first is that overproduced GSTs might protect the parasites against the effects of reactive oxygen species (ROS), thus increasing the susceptibility of mosquitoes, by neutralising the oxidative response to the *Plasmodium*. ROS have been shown to have a role in insect innate immune responses as a potent pathogen-killing agent. For example, in *An. gambiae* Kumar *et al.* (Kumar *et al*., 2003) demonstrated that ROS is involved in *P. berghei* melanotic encapsulation. However, Bahia *et al*. (Bahia *et al*., 2013) reported that the interactions between *An. aquasalis* and *P. vivax* do not follow the model of ROS-induced parasite killing, indicating that the role of ROS in immune response to *Plasmodium* infection could vary according the *Anopheles*-parasite system. Future studies are therefore needed to investigate the role of ROS in *An. funestus* response to *P. falciparum* infection. The second explanation is that resource-based trade-offs could have affected mosquito immune-competency. For instance overproduction of detoxifying enzymes, such as esterases or GSTs, is likely to deplete the resource pool, limiting the vector's ability to mount an immune response, therefore favouring the development of the parasite (Hall *et al*., 2009; Rivero *et al*., 2010). Considering this latter hypothesis, it could be likely that the low prevalence of infection in the RR genotype compared to the two others could have resulted in higher mortality before day 7 of individuals with a high load of infection. In fact mosquitoes of RR genotype may be less able to simultaneously maintain insecticide metabolic resistance and support development of a large amount of parasites, because of the limited host's energetic reserves. Likewise, Alout *et al.* (Alout *et al*., 2016) also reported that *Plasmodium* infection reduced the survivorship of females in both resistant Acerkis and Kdrkis strains of *An. gambiae sl*.

The effect of insecticide resistance on mosquito vector competence might differ according to the species and/or the resistance mechanism involved. In *An. gambiae*, Ndiath *et al.* (Ndiath *et al*., 2014) also reported high parasite burden in RR and RS than SS genotypes. In another study, Alout *et al.* (Alout *et al*., 2013)

showed that the *kdr* resistant allele is associated with reduced parasite burden in infected individuals at the oocyst stage, when compared to the susceptible strain. In *An. funestus,* Lo *et al.* (Lo & Coetzee, 2013) reported that pyrethroid resistant strain FUMOZ-R supported the lowest numbers of oocysts and sporozoites while the insecticide susceptible strain FUMOZ-BS produced highest sporozoite indices. In contrast, a recent study carried out by Tchouakui *et al.* (Tchouakui *et al.*, 2019) showed that a GST-mediated metabolic resistance to insecticides is associated with high *Plasmodium* infection in field resistant *An. funestus*. Altogether, observations from the present and previous mentioned studies showed that insecticide resistance, including GSTe2-based metabolic-based resistance, may affect the development of the malaria parasite in mosquito vectors. Considering the importance of *An. funestus* in malaria transmission and the wide distribution of insecticide resistance in this mosquito, these results are of great concern for the epidemiology of malaria in sub-Saharan Africa. However, because several host external and internal factors could influence *Anopheles-Plasmodium* interactions, additional work needs to be done to further assess the impact of insecticide resistance on malaria transmission and epidemiology. To minimize the impact of confounding factors, such work should be carried out on mosquitoes sharing similar genetic backgrounds. Finally, future experiments should also explore the impact of *GSTe2* on *Plasmodium* infection at sporozoite level as it is this stage that is transmitted to human through mosquito bites.

Data availability

Underlying data

Open Science Framework: Exploring the impact of glutathione *S*-transferase (GST)-based metabolic resistance to insecticide on vector competence of *Anopheles funestus* for *Plasmodium falciparum.* https://doi.org/10.17605/OSF.IO/JMYWF (Ndo, 2019)

This project contains the following underlying data:

- Gel1.tif (Representative gel image)
- Gel2.tif (Representative gel image)
- Gel3.tif (Representative gel image)
- Gel4.tif (Representative gel image)
- Gste2 gene sequence results.fas (Mosquito sampling and identification data)
- Raw data for infection and GSTe2 genotyping.xlsx (GSTe2 sequencing reads)

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Acknowledgments

We thank the participating children and their parents, for their involvement in this study; the local authorities, for their support.

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Reviewer Report 29 January 2020

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Majidah Hamid-Adiamoh
MRC Unit The Gambia at the London School of Hygiene and Tropical, Fajara, The Gambia
Mamadou Ousmane Ndiath
MRC Unit The Gambia at the London School of Hygiene and Tropical, Fajara, The Gambia

This paper entitled "**Exploring the impact of glutathione *S*-transferase (GST)-based metabolic resistance to insecticide on vector competence of *Anopheles funestus* for *Plasmodium falciparum*** presents on the influence of glutathione S-transferase resistance on vector competence of *Anopheles funestus* in two sites in Cameroon. The authors found no association between *GSTe2* variants and infection status of mosquitoes. It is a complex and difficult subject to quantify this relation because several factors and/or actions are involved in the vector-parasite relationship and each of them can negatively or positively affect the infection. From this point on and in view of the generalization of insecticide resistance, it becomes legitimate to ask this question. I am pleased to note that the authors have been able to appreciate the complexity of the issue and that, in the end, this is a preliminary study that should serve as a basis for moving forward, a point that they have developed well in the discussion.

This version 2 of the manuscript is very elaborate as it takes into account comments and observations of the previous reviewers. See below my specific comments on the different sections of the manuscript.

Abstract

- It is advisable to state specifically that the study was carried out in *An. funestus* populations in Yaounde, Cameroon. A different result may be obtained from other regions outside this belt. "Here, we investigated whether glutathione S-transferase-based resistance could influence *Plasmodium falciparum* development in *Anopheles funestus"* .

- Use of GSTe2 gene and its polymorphism should be italicized.

Methodology

- Under mosquito sampling and identification, is there a specific reason why the authors were silent about the frequency of mosquito collection? Was it only a one-time point collection? The overall number of mosquitoes (615 in Obout and 670 in Mibellon) gave an indication that mosquitoes could have been collected multiple times from each site which was not indicated.

- The authors indicated that: "*Progenies were pooled and reared to adulthood under standardized conditions*." Which progenies were pooled? Is it progenies from the study sites or from different collection periods? It is important for the authors to emphasize this as the pooling strategies may be interpreted otherwise.

Results

***Anopheles funestus* infection**

- There is a difference between the values shown in the text and the corresponding table. Precisely in Obout, the infection rate on the text is 42.74% whereas in Table 2 it is 41.93%, the same is true for the intensity of infection, again in Obout: 7.44+-1.20 in the text and 7.57+-1.22 in the table. Which is the right number? Table or text?

Distribution of L119F-GSTe2 resistance allele and *P. falciparum* infection

- This section looks confusing to me! In the text it was indicated that **218** and 185 mosquitoes were genotyped in Obout and Mibellon respectively while in the title of Figure 1 we are talking about **128** mosquitoes in Obout. Could you revise the numbers?

- The percentages on their own don't mean anything, especially with the point I just mentioned before, so I recommend that you give the values of (n) each time you give percentages.

A typo error: "A total of 218 and 185 mosquitoes was **genotyped**" not genotypes in Obout and Mibellon respectively.

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

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?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Entomologist, Malaria expert in molecular biology, insecticide resistance and vector biology.

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, however we have significant reservations, as outlined above.

Reviewer Report 10 January 2020

https://doi.org/10.21956/wellcomeopenres.16968.r36739

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Dari F. Da
Institut de Recherche en Sciences de la Santé, Bobo-Dioulasso, Burkina Faso

The manuscript in the version 2 included all comments and suggestion. The questions were clearly answered with more clarification. I give my approval for this article to be indexed.

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

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?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I'm malarialogist working mainly on the plasmodium human-mosquito transmission. My research interest focus on the Plasmodium/host/vector interactions (immunuo-molecular mecanisms) regulating parasite human-mosquito transmission in the experimental and field conditions. The final objective focus is to contribute to the development of malaria transmission blocking interventions using new tools (like altruistic vaccine, drugs, refractory mosquito, ...).

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 20 May 2019

https://doi.org/10.21956/wellcomeopenres.16431.r35232

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David P. Tchouassi
International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya

The authors investigated whether glutathione S-transferase-based resistance could influence *Plasmodium falciparum* development in *Anopheles funestus*. I commend the authors for the detailed piece of work. The experimental design and the findings are significant and address an important knowledge gap on the impact of insecticide resistance on relevant epidemiologic traits that can affect malaria control.

Minor weaknesses
The authors found a lower infection rate in the mosquitoes expressing resistance allele but higher oocyst intensity in this genotype. I think challenging the mosquitoes with the target insecticide would help explain these discrepancies. besides, I have reservations as to whether 40% and 60% infection rates in the RR and SS genotypes are significant statistically.

The authors use of very long sentences in especially in the discussion section. The thoughts could be communicated better in short simple sentences to improve on clarity. Kindly refer to the attached manuscript for comments and suggestions pertaining to typos or spelling mistakes

Others
Abstract:
Background: Use of 'interferes' sounds as if it is a known fact; better to say 'impact'.

The authors found lower *Plasmodium* infection rate in the mosquitoes expressing resistance allele but higher oocyst intensity in this genotype. In light of this, I am not convinced about the authors being emphatic that GSTe2-based metabolic resistance increases the mosquito permissiveness to *Plasmodium* infection as stated in the conclusion. I think this sentence needs to be revised.

Introduction:
Better to indicate the specific species in the *An. gambaie* sl where selection for resistance has been reported. This is a species complex and not sure such data exists for all members in this group. Also indicate clearly whether *An. funestus* refers to *An. funestus* s.s.
It is important to specify the mosquito species where the *GSTe2* mutation has been detected. Does it occur in all malaria vectors?

Materials and Methods
For the seasonal durations indicated, it may be helpful to indicate whether there may be year-to-year variations?
The authors should indicate the specific vector species in *An. gambiae sl* found in Mibellon; not all species in this group are vectors.

The sentence '*Anopheles funestus* was found to actively transmit malaria in the locality, with an infection rate of up to 3.7% needs revision; a pathogen is transmitted but not a disease.

Authors need to correct the description of RR, RS and SS on page 4 under L119F-GSTe2 mutation genotyping sub-title describing the DNA band sizes for each allele.

Authors should avoid writing words in the contracted forms e.g. didn't. this should be written in full and throughout the manuscript.
It is better for the authors to replace the word 'global' to 'overall' as used in the results and discussion sections of the manuscript.
Not sure about writing the all the p values to 4 dp.

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

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?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vector Biology and Ecology.

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.

Author Response 03 Oct 2019

Cyrille Ndo, Centre for Research in Infectious Disease (CRID), P.O. Box 13591, Yaoundé, Cameroon, Yaoundé, Cameroon

Abstract:
Reviewer's comment 1: Background: Use of 'interferes' sounds as if it is a known fact; better to say 'impact'.

Author's response 1: correction done.

Reviewer's comment 2: The authors found lower *Plasmodium* infection rate in the mosquitoes expressing resistance allele but higher oocyst intensity in this genotype. In light of this, I am not convinced about the authors being emphatic that GSTe2-based metabolic resistance increases

the mosquito permissiveness to *Plasmodium* infection as stated in the conclusion. I think this sentence needs to be revised.

Author's response 2: We added "possibly".

Introduction:
Reviewer's comment 3: Better to indicate the specific species in the *An. gambaie* sl where selection for resistance has been reported. This is a species complex and not sure such data exists for all members in this group. Also indicate clearly whether *An. funestus* refers to *An. funestus* s.s.
It is important to specify the mosquito species where the *GSTe2* mutation has been detected. Does it occur in all malaria vectors?

Author's response 3: The specific species have been indicated.

Materials and Methods
Reviewer's comment 4: For the seasonal durations indicated, it may be helpful to indicate whether there may be year-to-year variations?

Author's response 4: No, there is no year to year variation. We all know that seasons in a given region don't vary like that.

Reviewer's comment 5: The authors should indicate the specific vector species in *An. gambiae sl* found in Mibellon; not all species in this group are vectors**.**

Author's response 5: The specific species has been indicated.

Reviewer's comment 6: The sentence '*Anopheles funestus* was found to actively transmit malaria in the locality, with an infection rate of up to 3.7% needs revision; a pathogen is transmitted but not a disease.

Author's response 6: We changed malaria by "plasmodium parasites"

Reviewer's comment 7: Authors need to correct the description of RR, RS and SS on page 4 under L119F-GSTe2 mutation genotyping sub-title describing the DNA band sizes for each allele.

Author's response 7: We changed the sentece to " The size of the diagnostic band was 523 bp for homozygous resistant (RR) and 312 bp for homozygous susceptible (RS), while heterozygous (SS) showed the two bands.

Reviewer's comment 8: Authors should avoid writing words in the contracted forms e.g. didn't. this should be written in full and throughout the manuscript.

Author's response 8: We removed all the contracted forms.

Reviewer's comment 9: It is better for the authors to replace the word 'global' to 'overall' as used in the results and discussion sections of the manuscript.

Author's response 9: Global has been changed to overall.

Reviewer's comment 10: Not sure about writing the all the p values to 4 dp.

Author's response 10: We don't think that this could change the sense of the results

Competing Interests: I have no compteting interests

Reviewer Report 08 May 2019

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Dari F. Da
Institut de Recherche en Sciences de la Santé, Bobo-Dioulasso, Burkina Faso

The manuscript (Exploring the impact of glutathione S-transferase (GST)-based metabolic resistance to insecticide on vector competence of *Anopheles funestus* for *Plasmodium falciparum*) addresses an important question in the field epidemiology of malaria transmission. Fully elucidating major interactive parameters that are regulating Plasmodium transmission in malaria endemic areas is essential in the control of the disease. The content of this study, although superficial and required to be reformulated, appears as an important contribution in terms of the patterns between mosquito insecticide resistance and their vector capacity. Consistent design was presented with the appropriate methodology to achieve the objective mentioned. The global results were well described but some details remain to be completed (see comments attached in the pdf version of the manuscript). Some comparative analysis must be completed to clearly elucidate the relationship between the main parameters taking account some important variables. Because of this, superficial analysis in this version the discussion was likely based in large part on the previous studies.

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

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

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.

Reviewer Expertise: I'm malarialogist working mainly on the plasmodium human-mosquito transmission. My research interest focus on the Plasmodium/host/vector interactions (immunuo-molecular mecanisms) regulating parasite human-mosquito transmission in the experimental and field conditions. The final objective focus is to contribute to the development of malaria transmission blocking interventions using new tools (like altruistic vaccine, drugs, refractory mosquito, ...).

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 03 Oct 2019

Cyrille Ndo, Centre for Research in Infectious Disease (CRID), P.O. Box 13591, Yaoundé, Cameroon, Yaoundé, Cameroon

Reviewer's comment 1. change "mosquito" to "An. funestus"

Author's response 1: we changed ''mosquitoes'' to ''An. funestus mosquitoes''

Reviewer's comment 2. This last paragraph, essential to clearly highlight your problematic is unfortunately formulated with only 2 long sentences rendering confused hypothesis and objective. It requires to be rewritten with more details to fully express your scientific question.

Author's response 2: The paragraph has been rewritten.

Reviewer's comment 3. You clearly provided some details about Mibellon, one of your study sites, but the insecticide-resistance history of the major vectors in this locality is not mentioned here. Because your objective focus to explore the eventual impact of the genotype of the vectors in relation with their competence, it was expected to find the the mosquito insecticide background motivating your site choice. What comment could you provide here?

Author's response 3: You are right and we therefore added this information as follow:
"Both vectors have developed high levels of resistance to pyrethroids (deltamethrin and permethrin) and organochlorides (DDT) and moderate resistance to carbamate (bendiocarb) (Menze *et al*., 2018)."

Reviewer's comment 4. 30 out of 400 An funestus were used for gene sequencing (so less than 10% of your mosquito population). The 400 Anopheles (F1 generation) originating from more than thousand of field mothers (615+670) present evidently a various genetic background. The sequencing results of 30 out of 400 An funestus (a sampling in a sample) seem too small to be representative and could provide inappropriate tendencies. In the absence of reasonable proofs about this choice, an alternative solution would consist of sequencing more anopheles genes to increase your sample size if the DNA is always available.

Author's response 4: although analyzing 30 sequences of individuals out of 400 could seem small, it is insufficient provide appropriate tendances. In fact number of studies published in peer reviewed journals have generated very robust results by analyzing similar sample size or less per population. We can cite for example:

- Ndo *et al.* 2010 (malaria journal): Population genetic structure of the malaria vector Anopheles nili in sub-Saharan Africa (5 to 10 individuals analyzed per population)
- Menze *et al.* 2016 (Plos one): Multiple Insecticide Resistance in the Malaria Vector Anopheles funestus from Northern Cameroon Is Mediated by Metabolic Resistance Alongside Potential Target Site Insensitivity Mutations (5 dead and 5 alive sequenzed)
- Rireveron *et al.* 2014 (Genome biology): A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. (5-12 individuals analyzed per population)
- Weedall *et al*, 2019 (Sciences translational medicine): A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy. (15 individuals analyzed in each of the 10 countries across Africa).

Reviewer's comment 5. It is important to give the precision on the calculated mean: arithmetic or geometric? Mean estimated in the dissected population of Anopheles or only in those infected by Plasmodium oocyst? Depending on the study objective, evaluate the impact of intervention (vaccine, drug, ...) or just understand some biological mechanism in the parasite transmission the estimated oocyst mean and the mosquito population considered could be different.

Author's response 5: we now gave the precision in the text that it was arithmetic mean.

Reviewer's comment 6. If you have no conviction that several species were found in the locality, "An. gambiae sl" is more appropriated "than An. gambiae complex".

Author's response 6: We changed An. gambiae complex to An. gambiae sl.

Reviewer's comment 7. In the paragraph highlighted (however ... P:0.0003) it is understood than you try to compare the mosquito infection level between the 2 sites (Mibellon and Obout). This comparison has no sense for the reason that different human volunteers with of course various gametocytemia were used to perform the experiment. Gametocyte density, an important parameter in the mosquito infection could maybe help to argue this statement.

Author's response 7: We agree with the reviewer that use of different gametocyte carrier could lead to different infection parameters. The paragraph has been modified as follows:
Overall, the blood feeding rate of *An. funestus* through the artificial parafilm membrane was low compared to that of the *An. coluzzii* Ngousso strain used as control (80.42% - 100%) and didn't exceed 40% in all the cases (min - max: 1% - 38%). *Anopheles funestus* of both sites showed high susceptibility to natural *P. falciparum* isolates with 72.73% (8/11) and 77.78% (7/9) experiments yielding at least one infected mosquito in Obout and Mibellon, respectively. The overall infection rate was 69.73% Mibellon and 42.74% in Obout. By contrast infection intensity represented by mean and median oocyst load in midgut was moderate in Obout (mean: 7.44±1.20; median: 4) and low in Mibellon (mean: 2.88±0.18; median: 2)(Table 2).

Reviewer's comment 8. This last sentence (When the data ... significant) likely describes the opposite of what is observed in the table: From one experience to another and for each significant variations seem evident for both prevalence and oocyst intensity. Could you clearly include the comparative statistic tests performed to support your assertion?

Author's response 8: After having modified the paragraph above, we found necessary to remove the last sentence to make more sense

Reviewer's comment 9. For which reasons only experiments with 20% infection prevalence were considered for genotypic analysis?

Author's response 9: We use only experiments with at least 20% to be sure that gametocyte (blood) was really infective, but more importantly, this allowed us to have enough individual infected or non infected of different genotypes

Reviewer's comment 10. In this paragraph (The frequency ... RR vs RS), the authors must account for the origin of the mosquitoes (site) and the infectious doses (gametocytemia) in their analyses of the effects of resistance on parasite prevalence and intensity in mosquitoes. Because there are differences in resistance frequencies among villages (Fig 1), this observation has to be considered in the following statistical analyses of mosquito infection.
Such analysis will provide more information about what is qualified "contrasting results" and the discussion could be reformulated.

Author's response 10: In this paragraph, the analysis is not done according to the origin of mosquitoes but according to the genotype, irrespective of the origin. Since all the genotypes from a single experiment received the same infectious blood meal (same gametocytemia) and are all include in the analysis, we think that observations done are meaningful.

Competing Interests: I have no competing interests