RESEARCH ARTICLES

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

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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, interferes with 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 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. funestus mosquitoes to P. falciparum infection, by increasing its permissiveness to Plasmodium infection.
Keywords
Malaria, Insecticide resistance, Anopheles funestus, Plasmodium falciparum, metabolic resistance, GSTe2

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**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* s.l and *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 analysis 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 vector 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 tissue, for example, enzymatic modifications involved in metabolic resistance could render mosquito 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).

In *An. gambiae*, Alout et al. (Alout et al., 2014), demonstrated that the target-site mutations, ace-1 G119S and kdr L1014F conferring resistance to carbamates, DDT and pyrethroids respectively, are associated with increased *P. falciparum* infection prevalence and high sporozoite burden, probably enhancing mosquito vector competence and malaria transmission. Conversely, the lack of DNA-based markers for metabolic resistance has long prevented the assessment of the impact of this resistance mechanism on mosquito populations, including their ability to transmit *Plasmodium* parasites (vector competence). Progress made recently 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 (Rivero et al., 2014). Geographical distribution of this point mutation strongly correlated with insecticide resistance patterns across Africa and 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 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* showing 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 composed of woodlands with carpets of grass. 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* s.l and *An. funestus* are routinely found in the village, with the latter being the most abundant throughout the year. *Anopheles funestus* was found to actively transmit malaria 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 forest area of the Centre region (Southern Cameroon). The climate is of the equatorial Guinean, 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* s.l and *An. funestus* are the main vector species found in the village. High *Plasmodium* infection rates reaching 23.33% 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 parasitaemic 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 separate 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. Dissections were performed on all mosquitoes that were suspected to be infected. Carcasses were preserved at -20°C until DNA extraction was performed.

Throughout the experiments, we used the *An. coluzzii* Nguosso 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* Nguosso 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 thermalcycler (Model TC-E-48DA), in a reaction volume of 15 µl out using 10 µM of each primer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 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 detection of homozygous resistant (RR) at 523 bp, homozygous susceptible (RS) at 312 bp, and heterozygous (SS) with the presence of both 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 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, ddH₂O, 10X buffer A, 1.5 mM MgCl₂, 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.

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

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ndel_Gste2F</td>
<td>GGAATTCCATATGACCAAGCTAGTCTGTACAGGCT</td>
</tr>
<tr>
<td>Xbal_Gste2R</td>
<td>TCTACATCAAGCTTTAGCATTTTCCTT</td>
</tr>
<tr>
<td>L119F-Res</td>
<td>CGGGAAATGTCCGATTTCCGGATAGAA</td>
</tr>
<tr>
<td>L119F-Sus</td>
<td>CATTCTATTTCTACAGGAGGCTTACATC</td>
</tr>
</tbody>
</table>
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 mean and median number of oocysts in the midguts.

The geographical distribution of L119F-GSTe2 mutation was assessed by determining allelic and genotype frequencies in each study site. 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 between mosquitoes of different genotypes (RR, RS and SS). Prevalence of infection, mean, and median intensity of infection were computed and compared using the Fisher’s exact test and Mann-Whitney 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 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 from the An. gambiae complex (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) were 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 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. However, the global infection rate was significantly higher in Mibellon (69.73%) compared to Obout (42.74%) (Fisher’s test, P<0.0037). In contrast, infection intensity represented by mean and median oocyst load in midgut showed high values in Obout (mean: 7.44±1.20; median: 4) compared to Mibellon (mean: 2.88±0.18; median: 2) (Mann-Whitney U: 4992, P<0.0003). When the data was analyzed separately, these two parameters showed important variations across experiments, but the differences were no longer significant (Table 2).

Distribution of L119F-GSTe2 resistance allele and P. falciparum infection
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 suggesting high insecticide resistance in this site, 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 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: 0.0001 for RR vs SS; P<0.001 for RR vs SS; P:0.0329 for RR vs SS; P:0.0329 for RR vs SS) (Figure 2). As such, the odds ratio of being infected were significantly greater 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-Whitney 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
Table 2. Infection parameters in *An. funestus* from Obout and Mibellon.

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

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 and Mibellon. (SS: homozygous susceptible genotype, RS: heterozygous genotype, RR: homozygous resistant genotype. R: resistant allele, S: susceptible allele).
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=0.003) compared to the infected (π=0.005) 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.

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 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 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 in turn low in Mibellon (29.95%). This result, particularly in Mibellon, didn’t 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%) 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 highlights 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.
Table 3. Summary of GSTe2 sequence polymorphisms in infected and non-infected mosquitoes.

<table>
<thead>
<tr>
<th></th>
<th>Coding region</th>
<th>Non-coding region</th>
<th>Whole sequence</th>
</tr>
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<tr>
<td></td>
<td>Exon1 Exon2 Exon3 All</td>
<td>Intron1 Intron2 All</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>18 18 18 18 18</td>
<td>18 18 18 18 18</td>
<td></td>
</tr>
<tr>
<td>N seq</td>
<td>18 18 18 18 18</td>
<td>18 18 18 18 18</td>
<td></td>
</tr>
<tr>
<td>N indiv</td>
<td>9 9 9 9 9</td>
<td>9 9 9 9 9</td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>107 202 309 643 72</td>
<td>72 72 144 787</td>
<td></td>
</tr>
<tr>
<td>Poly sites</td>
<td>2 1 2 6</td>
<td>4 1 5 11</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>3 2 3 7</td>
<td>5 2 6 8</td>
<td></td>
</tr>
<tr>
<td>Hd</td>
<td>0.307 0.209 0.529 0.791</td>
<td>0.484 0.336 0.562 0.797</td>
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</tr>
<tr>
<td>pi</td>
<td>0.003 0.001 0.002 0.002</td>
<td>0.009 0.005 0.007 0.003</td>
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<tr>
<td>K</td>
<td>0.320 0.209 0.765 1.503</td>
<td>0.641 0.366 1.007 2.510</td>
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<tr>
<td>Fu Li D</td>
<td>-0.552 0.667 0.885 0.577</td>
<td>-0.070 0.667 -0.359 0.164</td>
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<tr>
<td>Fu Li F</td>
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<td>-1.008 0.708 -0.607 -0.121</td>
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<tr>
<td>Tajima's D</td>
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<td>-1.347 0.488 -0.974 -0.786</td>
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<tr>
<td>Non infected</td>
<td>16 16 16 16 16</td>
<td>16 16 16 16 16</td>
<td></td>
</tr>
<tr>
<td>N seq</td>
<td>16 16 16 16 16</td>
<td>16 16 16 16 16</td>
<td></td>
</tr>
<tr>
<td>N indiv</td>
<td>8 8 8 8 8</td>
<td>8 8 8 8 8</td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>107 202 309 643 72</td>
<td>72 72 144 787</td>
<td></td>
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<tr>
<td>Poly sites</td>
<td>1 2 3 6</td>
<td>3 6 9 15</td>
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<tr>
<td>H</td>
<td>2 2 4 6</td>
<td>4 4 6 9</td>
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<td>Hd</td>
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<td>0.517 0.442 0.675 0.883</td>
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<tr>
<td>pi</td>
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<td>0.008 0.021 0.014 0.005</td>
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<tr>
<td>K</td>
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<td>Fu Li D</td>
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<td>Fu Li F</td>
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<tr>
<td>Tajima's D</td>
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<td>-1.056 -0.662 -0.919 -0.740</td>
<td></td>
</tr>
</tbody>
</table>

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.
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 samples. We used the parafilm-glass feeding system for experimental infection of *An. funestus*, and this shows that 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 competent to develop the malaria parasite hence its major role in the transmission of this disease in sub-Saharan African settings. By detecting higher global infection rate in Mibellon, but higher infection intensity in Obout, our results didn’t 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 ecolimatic 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. Though, it could have been also interesting to examine this impact at sporozoite level, which is the parasite stage transmitted to humans, 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 last 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, due to their inability to simultaneously maintain insecticide metabolic resistance and support development of a large amount of parasites, because of the limited host’s energetic reserves. Otherwise, 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 dense infection in RR and RS than SS genotypes, while 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. (submitted) showed that a GST-mediated metabolic resistance to insecticide 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 in 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.
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)

References


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http://www.doi.org/10.17605/OSF.IO/JMYW


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Data availability

Underlying data


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

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