### A marker of glutathione S-transferase-mediated resistance to insecticides is

1. **associated with higher *Plasmodium* infection in the African malaria vector**
2. ***Anopheles funestus***

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# Abstract

1. Metabolic resistance to insecticides is threatening malaria control in Africa.
2. However, the extent to which it impacts malaria transmission remains unclear. Here,
3. we investigated the association between a marker of glutathione S-transferase
4. mediated metabolic resistance and *Plasmodium* infection in field population of
5. *Anopheles funestus* s.s. in comparison to the A296S-RDL target site mutation*.*
6. The 119F-GSTe2 resistant allele was present in southern (Obout) (56%) and
7. central (Mibellon) (25%) regions of Cameroon whereas the 296S-RDL resistant allele
8. was detected at 98.5% and 15% respectively. The whole mosquito *Plasmodium* and
9. sporozoite infection rates were 57% and 14.8% respectively in Obout (n=508) and
10. 19.7% and 5% in Mibellon (n=360). No association was found between L119F-
11. GSTe2 genotypes and whole mosquito infection status. However, when analyzing
12. oocyst and sporozoite infection rates separately, the resistant homozygote 119F/F
13. genotype was significantly more associated with *Plasmodium* infection in Obout than
14. both heterozygote (OR=2.5; P=0.012) and homozygote susceptible (L/L119)
15. genotypes (OR=2.10; P=0.013). In contrast, homozygote RDL susceptible
16. mosquitoes (A/A296) were associated more frequently with *Plasmodium* infection
17. than other genotypes (OR=4; P=0.03). No additive interaction was found between
18. L119F and A296S. Sequencing of the *GSTe2* gene showed no association between
19. the polymorphism of this gene and *Plasmodium* infection.
20. Glutathione S-transferase metabolic resistance is potentially increasing the
21. vectorial capacity of resistant *An. funestus* mosquitoes. This could result in a possible
22. exacerbation of malaria transmission in areas of high GSTe2-based metabolic
23. resistance to insecticides.

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1. **Keywords**: Malaria, insecticides, metabolic resistance, Glutathione S-transferase,
2. *Anopheles funestus*, *Plasmodium*

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### Background

1. Malaria control in Africa mainly relies on insecticide-based interventions such
2. as long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS)1. A
3. significant decrease in the incidence of malaria was reported between 2000 and
4. 2015, with about 70% of this success credited to insecticide-based vector control
5. tools1. However, the emergence of insecticide resistance in vector populations
6. resulting from widespread use of insecticides in public health, combined with
7. pesticide use in agriculture, is a major problem that is jeopardizing the control of
8. malaria2. The two main resistance mechanisms are target-site resistance (e.g.
9. knockdown resistance, *kdr*) and metabolic resistance through over-expression of
10. detoxification genes (e.g. cytochrome P450s, glutathione S-transferases and
11. esterases)3. Insecticide resistance genes are often associated with pleiotropic effects
12. on mosquito life-history traits4,5 which can modify their capacity to transmit parasites
13. to different hosts4,6. The fitness cost of resistant alleles could affect various vector
14. life-history traits, such as adult longevity, biting behavior, and vector competence7,
15. which are important components of the vectorial capacity to transmit pathogens.
16. However, despite the widespread distribution of resistance, its impact on malaria
17. transmission remains unclear in many malaria vectors including *Anopheles funestus*.
18. This is particularly true for metabolic resistance mechanisms since no molecular
19. markers were previously available to assess such an impact, in contrast to target-site
20. resistance (such as knockdown resistance *kdr*) for which DNA-based diagnostic
21. tools have been available for many years 8.
22. A better understanding of resistance mechanisms including metabolic
23. resistance and, more importantly, their impact on vector life traits and disease
24. transmission, is essential to design successful resistance management strategies 9.
25. A decrease in the ability of resistant mosquitoes to transmit malaria may mean
26. insecticide resistance is not detrimentally impeding the control of this disease7.
27. Conversely, if insecticide resistance increased the ability of resistant mosquitoes to
28. infect humans, this would lead to increased malaria transmission. Only a few studies
29. mostly, in *An. gambiae* s.s, have investigated the impact of resistance on vectorial
30. capacity4,6,10,11. For example, the study of Kabula *et al.* (2016) in Tanzania based on
31. the target-site resistance *kdr* marker11, showed that the infection of field populations
32. of *An. gambiae* s.s. by *Plasmodium* parasites was significantly associated with *vgsc-*
33. *1014* point mutations. Insecticide resistance was recently shown to affect the vector
34. competence of this same mosquito species for *P. falciparum* field isolates as a higher
35. prevalence of infection was observed for mutations associated with insecticide
36. resistance10. The impact of resistance on vectorial capacity has yet to be examined
37. for metabolic resistance which is the most common resistance mechanism in
38. mosquitoes. Metabolic resistance has consistently been reported to be the main
39. driver of pyrethroids and DDT resistance in the malaria vector *An. funestus*. No *kdr*
40. mutation has been detected so far in this species 12 despite the widespread report of
41. insecticide resistance in *An. funestus* populations across Africa.
42. Indeed, pyrethroid resistance has been reported in various *An. funestus*
43. populations including southern [Mozambique13,14, Malawi15,16], eastern [Uganda and
44. Kenya17,18 and Tanzania 19], central [Cameroon 20,21], and western Africa [Benin 22,23,
45. Ghana 24,25, Senegal26 and Nigeria23]. Noticeably, resistance to pyrethroids and DDT
46. in these populations is consistently conferred by detoxification enzymes including
47. glutathione S-transferases (GSTs) and cytochrome P450s. The predominance of
48. metabolic resistance in this species makes it suitable to investigate the impact of
49. metabolic resistance on malaria transmission. The detection of a single amino acid
50. change (L119F) in the glutathione S-transferase epsilon 2 (GSTe2) gene conferring
51. DDT/pyrethroid resistance in *An. funestus*27, further offers the opportunity to assess
52. this impact. In addition, the presence of target site mutations in *An. funestus* such as
53. the A296S-RDL associated with dieldrin resistance20 and N485I-Ace1 mutation
54. associated with bendiocarb resistance28 also allows to compare the effect of
55. metabolic resistance to that caused by target-site resistance on vectorial capacity of
56. this vector.
57. Therefore, to assess the potential impact of metabolic resistance on malaria
58. transmission, we investigated the association between the L119F-GSTe2 metabolic
59. resistance marker and the natural infection of *Plasmodium* parasites in two pyrethroid
60. and DDT resistant *An. funestus* populations from Cameroon. We established that the
61. 119F-GSTe2 resistance allele is significantly associated with *Plasmodium* infection in
62. resistant mosquitoes.
63. ***Results***

### Field collection and mosquito species identification

1. One thousand blood-fed female mosquitoes were collected in Obout and
2. 1,147 in Mibellon after a week of collection in each site and in at least ten houses
3. randomly selected in each village. Molecular identification of mosquitoes collected in
4. both localities revealed that 95% of the mosquitoes belonged to the *An. funestus*
5. group. The remaining 5% of mosquitoes were from *An. gambiae* species complex.
6. Nearly all the *An. funestus* mosquitoes belonged to *An. funestus s.s* as only one
7. mosquito belonging to another species of the *An. funestus* group (*An. leesoni)* was
8. detected in Mibellon.
9. **Infection rate of *An. funestus* by *Plasmodium* parasites**
10. In Obout, a total of 508 females (whole mosquitoes) randomly selected from
11. the field collected individuals were tested for *Plasmodium* infection. The overall
12. *Plasmodium* infection rate was very high in this locality with a total prevalence of
13. 57.1% (Figure 1a). Among the mosquitoes tested, 23% (119/508) were infected with
14. *P. falciparum* (falcip+), 19% (95/508) were infected with *P. ovale/vivax/malariae*
15. (OVM+), while 14.7% (76/508) were co-infected with both falcip+ and OVM+
16. (Table1). In addition, the head plus thorax and abdomen were analyzed separately in
17. 81 field-collected female mosquitoes to assess the proportion of mosquitoes
18. harboring the infective stage of the parasite (sporozoite) and those having the
19. oocysts. This is because sporozoites are predominantly present in the salivary glands
20. of mosquitoes. TaqMan assay revealed a sporozoite infection rate of 14.8% (12/81)

143 including 9.9% (8/81) falcip+, 2.5% (2/81) falcip+/OVM+ and 2.5% (2/81) OVM+.

1. Oocysts were detected in 30.8%(25/81) mosquitoes including 19.7% (16/81) falcip+,
2. 3.7% (3/81) falcip+/OVM+ and 7.4% (6/81) OVM+. The nested PCR performed on all
3. the infected mosquitoes confirmed all the 16 falcip+ by Taqman (Figure S1a)
4. whereas the three falcip+/OVM+ mosquitoes were co-infected with *P. falciparum* and
5. *P. malariae*. Out of 18 OVM+ by Taqman, 14 were infected with *P. malariae* (Figure
6. S1b) and the four remaining were not confirmed29. This observation indicates that in
7. this locality, *P. falciparum*, and *P. malariae* are in circulation.
8. In Mibellon, out of the 360 whole mosquitoes tested (randomly selected from
9. the total mosquitoes collected), 19.7% (71/360) were infected with *Plasmodium*

153 parasites including 10.8% (39/360) falcip+, 4.2% (15/360) OVM+, and 4.7% (17/360)

1. co-infection falcip+/OVM+ (Figure 1b)**.** Among the 60 mosquitoes dissected for
2. head/thorax and abdomen, 20% (12/60) were oocyst-positive and 5% (3/60)
3. sporozoite-positive with 3.3% (2/60) falcip+, 0% (0/60) falcip+/OVM+ and 1.7% (1/60)
4. OVM+. The nested PCR validation of the TaqMan assay for oocyst positive
5. mosquitoes confirmed all the six falcip+ whereas the two co-infected (falcip+/OVM+)
6. were confirmed as *P. falciparum* and *P. malariae-*positive. From the four OVM-
7. positive by Taqman assay, two were infected with *P. ovale and* two with *P. malariae*
8. showing that *P. falciparum, P. ovale* (Figure S1c) and *P. malariae* are all present in
9. Mibellon.
10. In both locations, a significant difference was found between mosquitoes
11. harboring the oocyst stage of the parasite and those with the infective sporozoite
12. stage (χ2=5.82; P=0.01 in Obout and χ2=6.12; P=0.01 in Mibellon).

### Association between L119F-GSTe2 mutation and total *Plasmodium*

1. **infection**
2. In total, 174 whole mosquitoes (infected and uninfected randomly selected
3. from the 508 tested above) from Obout were genotyped for the L119F-GSTe2
4. mutation (Table 2). All genotypes were successfully detected and later validated by
5. direct sequencing, supporting the robustness of the new designed Allele Specific-
6. PCR assay (Figure 2a-b). The 119F-GSTe2 resistant allele was found at a frequency
7. of 56.8% when combining both infected and uninfected mosquitoes. For infected
8. mosquitoes, 33.7% were 119F/F homozygous resistant, 47.2% L119F-RS
9. heterozygote, and 19.1% L/L119 homozygote susceptible (Figure 3a). A similar
10. distribution of the three genotypes was observed for uninfected mosquitoes (x2 =

178 0.34; P=0.82) with 31.7% for 119F/F, 49.4% for L119F-RS and 18.8% for L/L119

1. susceptible genotype. The lack of significant correlation between L119F-GSTe2
2. genotypes and whole mosquito *Plasmodium* infection was further supported by odds-
3. ratio estimates (Table 2).
4. In Mibellon, 184 whole mosquitoes randomly selected out of the 360 field-
5. collected females tested above were successfully genotyped. The overall frequency
6. of 119F-GSTe2 resistant allele was 26.3% in both groups of mosquitoes (infected
7. and uninfected). No difference was found in the distribution of the L119F-GSTe2
8. genotypes between infected and uninfected mosquitoes (*X2*=0.1 P=0.95) (Figure 3b)
9. (Table 2).

### Association between L119F-GSTe2 and oocyst and sporozoite infection

1. **rates**
2. In Obout, the distribution of L119F-GSTe2 genotypes in mosquitoes found
3. positive for oocysts by TaqMan was as follows: 28% (7/25) homozygous resistant
4. (119F/F), 40% (10/25) heterozygotes (L119F-RS) and 32% (8/25) homozygous
5. susceptible (L/L119F) (Figure 3c). No significant difference was observed in the
6. distribution of L119F-GSTe2 genotypes between infected and uninfected mosquitoes
7. (*X2*=3.58 P=0.17) (Table 3). At the sporozoite stage, 50% (6/12) of infected
8. mosquitoes were homozygous resistant, 33% (4/12) were heterozygotes and 17%
9. (2/12) were homozygous susceptible (Figure 3d) (Table 3). Contrary to the oocyst
10. stage, a significant difference was observed in the distribution of L119F-GSTe2
11. genotypes (using the proportions) between infected and uninfected mosquitoes
12. (X2=9.79 P=0.007). Assessing the odds-ratio between sporozoite infected and
13. uninfected mosquitoes showed that, homozygous resistant mosquitoes were
14. significantly more likely to be infected with sporozoites compared to both
15. heterozygote (OR=2.10; IC 95%: 1.11–3.97; P=0.013) and homozygous susceptible
16. (OR=2.46; IC 95%: 1.15–5.26; P=0.012) mosquitoes. There was no difference
17. between heterozygote and susceptible mosquitoes (OR=1.17; P=0.41) (Table 4).
18. In Mibellon, the 20% (12/60) of mosquitoes positive for oocysts were
19. comprised of 8.3% (1/12) 119F/F homozygous resistant, 58.3% (7/12) L119F-RS
20. heterozygotes and 33.3% (4/12) L/L119 homozygous susceptible (Figure 3e). A
21. significant difference was observed in the distribution of L119F-GSTe2 genotypes
22. between infected and uninfected mosquitoes (*X2*=13.05 P=0.001) with L119F-RS
23. heterozygote mosquitoes the most often infected (Table 3). Assessing the odd ratio
24. showed no difference between the two groups (Table 4). At the sporozoite stage,
25. only 5% (3/60) of mosquitoes were sporozoite positive and all were genotyped to be
26. homozygous susceptible (Figure 3f) (Table 3). No further comparisons were done
27. because of low sample size.

### Association between A296S–RDL mutation and *Plasmodium* infection

1. The RDL mutation was genotyped using gDNA extracted from 100 and 142
2. whole female mosquitoes from Obout and Mibellon respectively. In Obout, all
3. mosquitoes carried the resistant allele with a very high frequency of homozygous
4. resistant 296S/S genotypes (97%). In contrast, in Mibellon, only 22 (15.5%) of the
5. mosquitoes examined had the A296S-RDL mutation including 4 (2.8%) 296S/S
6. homozygous resistant, 18 (12.7%) A296S-RS heterozygotes and 120 homozygote
7. susceptible (Figure 4a). In addition, the A/A296 homozygous susceptible were
8. present in a higher proportion among the infected mosquitoes (90%) compared to
9. uninfected mosquitoes (79%). Assessment of the odd ratio demonstrated that
10. mosquitoes that were A/A296 homozygous susceptible were more likely to be
11. *Plasmodium* positive compared to other genotypes (OR=4; IC 95%: 1.24–12.86;

230 P=0.03) (Table S1).

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### Combined impact of L119F-GSTe2 and A296S-RDL mutations on

1. ***Plasmodium* infection**
2. In total, 46 samples of each batch (infected and uninfected mosquitoes) were
3. used to assess the combined impact of the two resistance mechanisms on
4. *Plasmodium* infection in in *An. funestus* mosquitoes (Figure 4b). The most prevalent
5. combinations between both groups were (SS/SS), (RR/SS), (RS/SS) for *GSTe2*/*RDL*.
6. The SS/RR and RR/RR combinations were present only among the infected
7. mosquitoes whereas the RR/RS and RS/RS combinations were observed only
8. among the uninfected mosquitoes. Nevertheless, no statistically significant
9. differences were detected (χ2=10.5; P=0.161). A significant difference was observed
10. when comparing the odds ratio at the sporozoite stage between RR/RR vs SS/RR
11. (OR=INF; P˂ 0.0001) and RS/RR vs SS/RR (OR=INF; P=0.003) indicating that
12. double homozygote mosquitoes (RR/RR) were more likely to be infected. However,
13. no significant difference was observed at oocyst stage P≥0.16) (Table S2). This
14. supports the role of the L119F-GSTe2 allele in the ability of the mosquitoes to
15. develop the parasite until the infective stage.

### Association between GSTe2 polymorphism and *Plasmodium* infection

1. **Genetic diversity of GSTe2:** The full length of the *GSTe2* gene (881bp) was
2. successfully sequenced in 26 whole mosquitoes from Mibellon including 11 infected
3. and 15 uninfected (Figure 5a). The genetic diversity parameters are given in Table
4. S3, according to the status of infection and genotypes. Overall, 23 polymorphic sites
5. defining 28 haplotypes were detected corresponding to the haplotype diversity of
6. 0.96. Heterozygous and uninfected mosquitoes showed at lower number of
7. polymorphic sites (3) with only 3 haplotypes (hd: 0.83). The overall nucleotide
8. diversity was 0.005 with an average number of differences between nucleotides
9. estimated at 4.19 showing less differences between the sequences examined. In
10. addition, negative values were obtained for Fu and Li Tajima and F \* tests in many
11. cases.
12. **Distribution of haplotypes and phylogeny**: Analysis of the haplotype
13. network of the *GSTe2* gene based on L119F genotypes of the infection status shows
14. that there are five major haplotypes (H1, H2, H3, H4 and H5) responsible for the
15. differentiation of haplotypes in this *An. funestus* field population. The ancestral
16. haplotype (H1) as well as the haplotypes (H2, H3 and H4) were identified in
17. mosquitoes with 119Fresistant allele. However, haplotypes H1 and H2 were found
18. only in uninfected mosquitoes whereas haplotypes H3 and H4 were present in both
19. infected and uninfected mosquitoes (Figure 5b-c). Moreover, the H5 haplotype was
20. found to be specific for mosquitoes with the L119 susceptible allele for both infected
21. and uninfected individuals (Figure 5d-e). Similarly, analysis of phylogeny between the
22. haplotypes identified did not reveal any haplotype groups associated with a specific
23. infection status. However, there was a global clustering according to the alleles
24. associated with the L119F mutation of the *GSTe2* gene (Table S3). This suggests
25. that there is no association between the polymorphism of the *GSTe2* gene and
26. infection by *Plasmodium* parasite.

### Discussion

1. Little information exists on the impact of metabolic resistance on the ability of
2. mosquitoes to transmit *Plasmodium* parasites. This study is among the first to assess
3. the association between metabolic resistance and vectorial capacity of natural
4. populations of malaria vectors. We took advantage of the recent detection of the
5. glutathione S-transferase L119F-GSTe2 marker in *An. funestus* to investigate the
6. relationship between metabolic resistance and vectorial capacity in field collected
7. mosquitoes. Mosquitoes used were collected from the same population at the same
8. time for them to share a common genetic background but differ only by the presence
9. of the resistant alleles to specifically discriminate this trait. Thus, any difference
10. observed between the three genotypes would directly be associated with the
11. insecticide resistance alleles.
12. **Role of *An. funestus* in malaria transmission**
13. *An. funestus* s.s*.* was found to be the most abundant mosquito species from
14. the indoor collection in the two study sites correlating with the indoor feeding/resting
15. behavior of this species and supported by the presence of permanent large pools of
16. water in both locations. *An. funestus* s.s. is playing a major role in malaria
17. transmission in these areas with a very high infection rate recorded at all stages
18. including sporozoite infection rates (14.8% in Obout and 5% in Mibellon) in field
19. collected females’ mosquitoes. The high infection rate observed in Obout and
20. Mibellon for *An. funestus* s.s. is similar to high levels of infection rates recorded
21. previously for this species across the continent such as 20%30 and 50%31 observed
22. in Burkina Faso, 13.6%32 and 18%33 observed in Benin and 12.5% in Ghana25.
23. Although, some of the variations between these rates could be attributed to the
24. differences in the detection methods used (TaqMan, ELISA and Nested-PCR), the
25. consistently high levels of infection support a high vectorial capacity of *An. funestus*
26. across the continent. This is of great concern for malaria control as it shows that
27. despite ongoing control interventions, the level of malaria transmission could remain
28. high in areas where *An. funestus* is the dominant vector. In this study, we noticed that
29. *P. falciparum* was the predominant malaria parasite in both study sites. However, the
30. detection of other malaria parasites, although at lower frequencies, is an indication
31. that control and elimination efforts should not ignore other *Plasmodium* species
32. especially *P. malariae*.

### Distribution of L119F-GSTe2 metabolic and A2926S target site resistance

1. **markers**
2. It was previously demonstrated that a single amino acid change (L119F) in the
3. over-expressed GSTe2 enzyme in *An. funestus s.s* confers resistance against DDT
4. and cross-resistance to permethrin in West Africa 27. The L119F mutation was
5. detected in both localities with a higher frequency of the resistant allele in Obout. This
6. resistance mechanism could have been selected in this population either by past
7. DDT based IRS34 or by the scale up of pyrethroid-based LLINs. The presence of this
8. resistance marker at high frequency in Obout supports previous observation in
9. northern Cameroon (Gounougou) by Menze et al.21 suggesting that this mutation is
10. strongly associated with DDT and permethrin cross resistance in Western and
11. Central Africa 21,27,33**.** In Mibellon, the 119F-GSTe2 mutation was found at a lower
12. frequency for the 119F-GSTe2 resistant allele (26.3%). Two hypotheses may explain
13. this: either the mutation was recently introduced in that population or that a recent
14. insecticide selection pressure is favoring its presence now.
15. The A296S-RDL mutation in the GABA receptor gene associated with dieldrin
16. resistance is fixed in the Obout population. In contrast, this mutation was found only
17. at very low frequency in Mibellon. The high frequency of 296S resistant allele in *An.*
18. *funestus* field populations from Obout is intriguing since cyclodienes are no longer
19. used for vector control in Cameroon. It has previously been shown that dieldrin
20. resistant mosquitoes exhibit significant fitness costs including behaviour and mating
21. competitiveness35,36 that should lead to a decrease in the frequency of the resistant
22. allele from the population overtime. Therefore, reversal of the resistance was
23. expected in this field population in the absence of dieldrin selection pressure. The
24. persistence of this dieldrin resistance marker in the *An. funestus* field population
25. from Obout may be associated with the use of pesticides in the agricultural sector
26. such as fipronil or lindane acting on same GABA receptor as dieldrin 37. A population
27. of *An. gambiae* fully resistant to dieldrin (100% RR) was reported in 1961 in
28. Mbalmayo, a location of south Cameroon close to Obout 38. This suggests also that
29. the A296S resistant allele in *An. funestus* population in Obout could have become
30. fixed before the removal of dieldrin as a vector control tool, thereby limiting the
31. possibility of reversing dieldrin resistance.

### Impact of L119F-GSTe2 metabolic and A2926S target site resistances on

1. ***Plasmodium* infection**
2. We did not detect any significant differences between L/L119 homozygote
3. susceptible, L119F-RS heterozygote and 119F/F homozygote resistant genotypes
4. and *P. falciparum* oocyst infection. However, mosquitoes with the A/A296 *RDL*
5. susceptible genotype were found to be more often infected with *Plasmodium*
6. parasites. It has previously been demonstrated that insecticide resistance
7. mechanisms may alter the vector competence of the mosquito by affecting parasite
8. development or susceptibility of the host to infection. This trend was not observed in
9. this study for the A296S-RDL mutation. In a study assessing the link between
10. insecticide resistance and vector competence, Alout and collaborators demonstrated
11. that target site mutations (*kdr* and *ace-1R*) increased the prevalence of *P. falciparum*
12. infection in pyrethroid resistant *An. gambiae* compared to their susceptible
13. counterparts10. However, despite a higher prevalence of infection, the *kdr* resistant
14. mosquito strain was found to harbour lower malaria parasite (oocyst) load10. Many
15. factors can influence the ability of mosquitoes to be successfully infected by
16. *Plasmodium* and harbour the parasites throughout their developmental stages until
17. the sporozoite stage. Therefore, pleiotropic effects of insecticide resistance, immunity
18. activation and other fitness related traits may be altered. McCarroll *et al* reported that
19. insecticide resistance in *Culex quinquefasciatus* mosquitoes had negative effect on
20. the parasitic worm *Wuchereria bancrofti*, which causes human lymphatic filariasis4,39.
21. Furthermore, vector immunity could also be affected by insecticide resistance7. A
22. possible link between resistance and mosquito immunity was observed when a gene
23. driving pyrethroid resistance was up-regulated in the mosquito mid-gut infected with
24. malaria parasites40. *An. gambiae* with metabolic resistance has also been shown to
25. have increased infection rates compared to controls10. However, esterase
26. metabolism or ace-1 mutation (target site resistance) did not appear to effect the
27. infection rates or parasitic load in *Culex pipiens* mosquitoes6. Oxidative stress is part
28. of the mosquito’s immune response against *Plasmodium* but may be neutralised by
29. overproduction of GSTs. Previously, GST resistance mechanisms were shown to
30. protect tissues from oxidative damage in plant hoppers and increase longevity in fruit
31. flies 41. Therefore, neutralising oxidative stress could potentially predispose
32. mosquitoes to higher parasite infection.

### Assessing the association between the L119F-GSTe2 mutation and the

1. **ability of mosquitoes to develop malaria parasites to the sporozoite stage**
2. The high sporozoite infection rate of 14.8% observed in Obout was similar to
3. recent observations in some GSTe2 related insecticide resistant populations of *An.*
4. *funestus* in Benin33 and in Democratic Republic of Congo42. On the other hand, it was
5. higher than the infection rates recorded in other pyrethroid resistant *An. funestus*
6. populations in southern Africa such as in Malawi (4.8%)43 or in many African
7. countries for other malaria vectors such as *An. gambiae*11,22. This high *Plasmodium*
8. infection rate in *An. funestus* highlights the active transmission of malaria in southern
9. Cameroon by this species. Furthermore, a significant association was found between
10. the 119F/F-GSTe2 resistant genotype and the presence of *P. falciparum* sporozoite
11. in *An. funestus* in Obout. The sporozoite infection rate in 119F/F homozygous
12. resistant mosquitoes was three times higher than that of the homozygous susceptible
13. mosquitoes. This suggests that parasites developed better in resistant mosquitoes
14. than in susceptible counterparts which should be a cause for concern as possession
15. of this resistance allele may potentially be allowing higher malaria transmission. A
16. similar result was previously found in *An. gambiae* s.s. for the target-site *vgsc*-
17. L1014S mutation11. The association between *Plasmodium* sporozoite infection and
18. GSTe2-based resistant mosquitoes observed in this study could be due to three main
19. possibilities. First, this could be caused by the phenotypic expression of L119F-
20. GSTe2 such that the 119F/F homozygous resistant mosquitoes could live longer due
21. to their ability to withstand exposure to insecticides in the field. In this case they are
22. more likely to allow the *Plasmodium* parasites to complete their extrinsic incubation
23. period compared to homozygous susceptible mosquitoes. This suggestion is
24. supported by the fact that glutathione S-transferases have been shown to be
25. associated with resistance27 and also to protect insect tissues from the damaging
26. effects of oxidative stress and extent life span of insects by increasing solubility and
27. excretion of free radicals44-47. The second possibility of the higher likelihood of
28. sporozoite infection in mosquitoes with GSTe2-119F/F genotype is that insecticide
29. resistance could alter mosquito immunity. Indeed, it is possible as suggested
30. previously 47 that the over-expression of GSTs in homozygous mosquitoes could be
31. protecting *Plasmodium* parasites against the damaging effects of reactive oxidative
32. species (ROS). These ROS are known as key component of the mosquito immune
33. responses against *Plasmodium* infection48. It is likely that over-expression of GSTs
34. may affect parasite development or susceptibility of the mosquito to infection by
35. neutralizing the oxidative response of the 119F/F mosquitoes to *Plasmodium* and
36. thus potentially increasing their susceptibility to infection. This will need to be fully
37. established possibly through experimental infection studies. Thirdly, the higher
38. *Plasmodium* infection rate seen in homozygous resistant 119F/F mosquitoes could
39. be due the potential reduction of immune-competence through a resource trade-off
40. between increased over-expression of GSTs and the mosquito’s immune response. It
41. has been shown that when certain energy resources are redirected towards the
42. production of large amounts of detoxification enzymes such as GSTs, a resource-
43. based trade-off is usually involved and affects the vector immuno-competence49. As
44. a result, there is likely to be a depletion of energy resources which limits the vector’s
45. ability to mount a sufficient immune response against *Plasmodium* leading to
46. increased infection in those resistant mosquitoes as observed in our study. However,
47. more studies are needed to establish the extent to which insecticide resistance
48. affects the mosquito’s vectorial capacity to confirm the impact of resistance on
49. malaria transmission.

### Conclusion

1. This study investigated the association between a molecular marker of GST-
2. mediated metabolic resistance and *Plasmodium* infection in natural populations of a
3. major malaria vector, *An. funestus*. The study revealed that mosquitoes that were
4. homozygous for the resistance allele were more likely to harbor *Plasmodium*
5. sporozoites. This suggests that the proliferation of this metabolic resistance marker
6. could exacerbate malaria transmission in the field and thus have important public
7. health consequences.

### Methods

1. **Study site and sample collection**
2. Mosquito collections were performed in Cameroon in May 2016 and February
3. 2017 in Obout (Southern Region, 3°28'17.0"N 11°44'09.4"E) and Mibellon
4. (Adamaoua Region, 6°46’N, 110 70’E) for one week per site. Prior to mosquito
5. collection, verbal consent was obtained from the village council chairpersons and
6. from each household representative. Indoor resting female mosquitoes were
7. collected using electric aspirators in both locations and transported to the insectary of
8. LSTM Research Unit at OCEAC in Yaoundé, Cameroon.

### DNA extraction

1. Genomic DNA (gDNA) was extracted via the LIVAK method 50. Following
2. extraction, NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, USA)
3. was used to determine the concentration and purity of the extracted gDNA before
4. storage at -20ºC.

### Species identification

1. The females used for oviposition were morphologically identified using the key
2. of Gillies and De Meillon51. Molecular identification was achieved through a cocktail
3. polymerase chain (PCR) reaction described by Koekemoer52 to determine species
4. composition of *An. funestus* group in the two study sites.
5. **Detection of *Plasmodium* parasites**
6. A TaqMan assay described by Bass et *al.* 29 was used to establish the
7. *Plasmodium* infection status of field collected mosquitoes. Two probes were used in
8. this assay. The first, labelled with FAM, detects *P. falciparum*, and the second,
9. labelled with VIC, to detect *P. vivax, P. ovale* and/or *P. malariae* (OVM). Firstly,
10. gDNA was extracted from the whole mosquitoes to assess the overall proportion of
11. *An. funestus* infected by *Plasmodium* parasites in the field. Secondly, another sets of
12. field collected mosquitoes were dissected in two parts: the abdomens, used for the
13. detection of *Plasmodium* infection at the oocyst stage, and the head plus thorax for
14. the assessment of sporozoite infection rate. Results of TaqMan assay were
15. confirmed by performing a nested PCR assay as previously described53.

### Genotyping of the L119F-GSTe2 mutation

1. The L119F-GSTe2 mutation previously shown to play a major role in DDT and
2. permethrin resistance in *An. funestus*27 was genotyped in F0 field-collected
3. mosquitoes using a newly designed allele-specific PCR (AS-PCR) diagnostic assay.
4. Two pairs of primers were needed for the AS-PCR (two outer and two inner primers).
5. Specific primers were designed manually to match the mutation and an additional
6. mismatched nucleotide was added in the 3th nucleotide from the 3′ end of each inner
7. primer to enhance the specificity. More details on the primer sequences are given in
8. Table S4. PCR was carried out using 10 mM of each primer and 1ul of genomic DNA
9. as template in 15 μl reactions containing 10X Kapa Taq buffer A, 0.2 mM dNTPs,
10. 1.5 mM MgCl2, 1U Kapa Taq (Kapa biosystems). The cycle parameters were: 1 cycle
11. at 95°C for 2 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1min and then a
12. final extension step at 72°C for 10 min. PCR products were separated on 2%
13. agarose gel by electrophoresis. This method detects homozygote resistant (119F/F)
14. at 523bp, homozygote susceptible (L119F-RS) at 312bp, and heterozygote (L/L119)
15. with both bands. Association between the *GSTe2* mutation and malaria transmission
16. potential was assessed by calculating the odds ratio of sporozoite infection rate
17. between the homozygous resistant (119F/F), heterozygote (L119F-RS) and
18. homozygous susceptible (L/L119) individuals compared to uninfected individuals,
19. with statistical significance was computed based on the Fisher’s exact probability
20. test.

### Association between the genetic diversity of the *GSTe2* gene and

1. ***Plasmodium* infection in *An. funestus***
2. The entire *GSTe2* gene of 881bp in *An. funestus* was amplified in 26 whole
3. mosquitoes [11 infected by *Plasmodium* parasites (both stages) and 15 non-infected].
4. Two primers; GSTe2F, 5’GGA ATT CCA TAT GAC CAA GCT AGT TCT GTA CAC
5. GCT 3’ and GSTe2R, 5’ TCT AGA TCA AGC TTT AGC ATT TTC CTC CTT 3’ were
6. used to amplify the gene in 15 μl reaction containing 10mM of each primer, 10X Kapa
7. Taq buffer A, 0.2 mM dNTPs, 1.5 mM MgCl2, 1U Kapa Taq (Kapa biosystems). PCR
8. conditions were 1 cycle at 95°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s,
9. 72°C for 1min and then final extension at 72°C for 10 min. PCR product was firstly
10. visualized on 1.5% agarose gel stained with Midori green dye (Nippon Genetics
11. Europe, Germany) and then purified using ExoSAP (Thermo Fisher Scientific,
12. Waltham, MA, USA) according to manufacturer recommendations and directly
13. sequenced on both strands. Sequences were visualized and corrected using BioEdit
14. v7.2.5 software 54. Alignment of these sequences was done using ClustalW Multiple
15. Alignment integrated in BioEdit 55. Genetic diversity parameters were assessed using
16. DnaSP v5.10.01 56 and MEGA v7.0.21 57 softwares.

### Genotyping of the A296S-RDL GABA receptor mutation

1. To compare the role of metabolic resistance to that of target-site resistance
2. mechanism, we genotyped the A296S-RDL mutation associated with dieldrin
3. resistance 58 in *Plasmodium* infected and uninfected mosquitoes. The A296S-RDL
4. mutation was genotyped using a protocol previously described by Riveron *et al*43.
5. Furthermore, the combined effect of harboring both alleles of A296S-RDL and
6. L119F-GSTe2 on the infection status of field collected mosquitoes was also
7. assessed.

### Additional Information

1. **Competing interests**
2. The authors declare that they have no competing interests.
3. **Author contributions**: C.S.W. conceived and designed the study; M.C.C., J.
4. M.R., and M.T. carried out the sample collection and performed WHO susceptibility
5. assays; M.T., M.C.C., M.J.W., C.K.K., M.K and M.T. performed the Molecular
6. analyses; M.T., N.A.A., C.K.K., C.N., J.M.R. and C.S.W. analyzed the data. M.T.,
7. J.M.R. and C.S.W. wrote the manuscript with contribution from other co-authors. All
8. authors read and approved the final manuscript.

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### Figure legends

**Figure 1: *Plasmodium* infection pattern in Obout (A) and Mibellon (B)** falcip+, infection by *Plasmodium falciparum*; falcip+/OVM+, Co infection by *Plasmodium falciparum* and *P.ovale/vivax/malariae*; OVM+, infection by *P. ovale/vivax/malariae.*

### Figure 2: Design of a new AS-PCR for genotyping the L119F-GSTe2 mutation.

a) Amplification by PCR of *GSTe2* gene in *An. funestus* s.s*.* and an overview of the polymorphism of the *GSTe2* gene at the L119F point mutation where Y represents the heterozygote genotype C/T. b) Agarose gel of AS-PCR to detect the L119F gste2 mutation in *An. funestus* s.s*.*. Top band 849 bp, fragment common of all genotypes; the middle (523bp) and the bottom (312bp), resistant and susceptible mosquitoes respectively; heterozygote mosquitoes, 523bp and the bottom 312bp fragments. M: Molecular ladder 100 bp; positive controls (S: homozygous susceptible, R : homozygous resistant and H : heterozygote) ; N: negative control; 1 – 15: samples genotyped (1, 6, 10: homozygous resistant; 7, 11: heterozygote; 2-5, 8, 12, 13, 15: homozygous susceptible; 9, 14: no amplification).

**Figure 3: Impact of the GSTe2 glutathione S-transferase metabolic resistance (L119F-GSTe2) on the infection and transmission patterns of *Plasmodium* parasites in natural *Anopheles funestus s.s*. populations** (a), (c) and (d); are whole mosquitoes, oocyst and sporozoite infection respectively in southern Cameroon (Obout); (b), (e) and (f); whole mosquitoes, oocyst and sporozoite infection respectively in Central (Mibellon).

**Figure 4: Impact of A296S-RDL target-site mutation on *Plasmodium* infection:** distribution of genotype (a) RDL and (b) combinations GSTe2 /RDL)) between infected and uninfected whole mosquitoes.

**Figure 5: Genetic diversity parameters of *GSTe2* in *An. funestus* s.s*.* from Mibellon in relation to *Plasmodium* infection.** a) haplotype network and b) phylogenetic tree (using a maximum likelihood method) between infected and uninfected mosquitoes; c) haplotype network and d) phylogenetic tree (using a maximum likelihood method) between 119F resistant allele and L119 susceptible allele.

**Table 1:** Status of infection by *Plasmodium* parasites in whole mosquitoes

***Plasmodium* infection**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Localities** | **N** | **Falcip+** | **Falcip+/ OVM+** | **OVM+** | **Total infection** |
|  |  | 119 (23 %) | 76(15 %) | 95(19 %) | 290 (57.1 %) |
| **Obout** | 508 | [20%-27%] | [12%-18%] | [15%-22%] | [52.7% - 61.3%] |
|  |  | 39 (11%) | 17(5%) | 15(4 %) | 71(19.7%) |
| **Mibellon** | 360 | [8%-14%] | [3%-7%] | [2%-7%] | [15.9% - 24.1%] |

**Abbreviations:** N, total number of mosquitoes tested; Falcip+, infection by *Plasmodium falciparum*; Falcip+/OVM+, Co-infection by plasmodium falciparum and *P.ovale/vivax/malariae*; OVM+, infection by *P. ovale/vivax/malariae*

**Table 2:** Distribution of L119F-GSTe2 genotypes according to *plasmodium* infection.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **phenotype** | **N** |  **L119F GSTe2 genotypes RR RS SS** | **Statistic test** | ***p value*** |  |
| **Infected** | 89 | 30 42 17 |  |  |  |
| **Obout non infected** | 85 | 27 42 16 | x2 = 0.34 | 0.82 |  |
| **% infection** |  | 52.6% 50% 51.5% |  |  |  |
| **Infected** | 41 | 2 18 21 |  |  |  |
| **Mibellon non infected** | 143 | 8 59 76 | x2 = 0.11 | 0.94 |  |
| **% infection** |  | 20% 23.3% 21.6% |  |  |  |

N, total number of mosquitoes successfully genotyped; RR, homozygous resistant; RS, heterozygous; SS, homozygous susceptible.

**Table 3:** Distribution of L119F-GSTe2 genotypes between mosquitoes infected with

*Plasmodium* and the prevalence of infection at both oocyst and sporozoite stage in

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Phenotype** | **N** | **RR** | **RS** | **SS** | **Statistic test** | ***P******value*** |
| **Obout** |
|  | Infected | **25** | 7 10 | 8 |  |  |
| **Oocyst** | Uninfected | **56** | 19 23 | 14 | x2 = 3.58 | 0.17 |
|  | **% infection** |  | **29.9% 30.3%** | **36%** |  |  |
|  | Infected | **12** | 6 4 | 2 |  |  |
| **Sporozoite** | non infected | **151** | 20 28 | 17 | x2 = 9.79 | 0.007\* |
|  | **% infection** |  | **23.1% 12.5%** | **10.5%** |  |  |
|  |  |  | **Mibellon** |  |  |  |
|  | Infected | **11** | 1 7 | 4 |  |  |
| **Oocyst** | non infected | **14** | 3 14 | 24 | x2 = 13.05 | 0.001\* |
|  | **% infection** |  | **10% 38.1%** | **18.75%** |  |  |
|  | Infected | **3** | 0 0 | 3 |  |  |
| **Sporozoite** | non infected | **49** | 4 21 | 25 | NA | NA |

Obout and Mibellon (Fisher Exact probability test based on the proportions).

**Table 4:** Assessment of the association of different L119F-GSTe2 genotypes with

*Plasmodium* infection status in Obout and Mibellon.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Whole mosquitoes** |  | **Oocyst infection** |  | **Sporozoite** | **infection** |
| **Genotypes** | **Odds ratio P-value** |  | **Odds ratio P-value** |  | **Odds ratio** | **P-value** |
|  |  |  | **Obout** |  |  |  |
| **RR vs RS** | 1.11 0.43 |  | 0.7 0.30 |  | 2.10 | **0.01\*** |
|  | (0.59– 2.07) |  | (0.41– 1.52) |  | (1.11 – 3.97) |  |
| **RR vs SS** | 1.06 0.52 |  | 0.64 0. 34 |  | 2.46 | **0.01\*** |
|  | (0.48-2.36) |  | (0.18 – 2.19) |  | (1.15– 5.26) |  |
| **RS vs SS** | 0.96 0.53 |  | 0.62 0.12 |  | 1.17 |  |
|  | (0.45 – 2.03) |  | (0.31 – 1.25) |  | (0.54 – 2.51) | 0.41 |
|  |  |  | **Mibellon** |  |  |  |
| **RR vs RS** | 0.77 0.47 |  | 0.67 0.33 |  | NA | - |
|  | (0.22– 2.74) |  | (0.22 – 2.01) |  |  |  |
| **RR vs SS** | 0.86 0.54 |  | 1.92 0.17 |  | NA | - |
|  | (0.25 – 3.01) |  | (0.66 – 5.95) |  |  |  |
| **RS vs SS** | 1.11 0.41 |  | 2.96 0.0002\* |  | NA | - |
|  | (0.63 – 1.92) |  | (1.62 – 3.58) |  |  |  |



Total= 508

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Total= 290

* **Uninfected**
* Infected
	+ Falcip+
	+ OVM+
	+ Falcip+/OVM+

**Uninfected**

Infected

* + - Falcip+
		- OVM+
		- Falcip+/OVM+

Total =360 Total= 71

849bp

.....

523pb

312pb

L119F-GSTe2

I

**a**

460

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**M** S R H N 1 2 3 4 5 6 7 8 9 10 11 12 13 14

1. Whole Mosquito Obout C

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Oocyst infection d Sporozoite infection

Infected Non infected

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Whole mosquito Mibellon

Infected Uninfected Infected Non infected

Oocyst infection Sporozoite infection

,m ss

* RS
* RR

Infected Non infected

Infected Non infected Infected Non infected

(a)

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**80**

* RR
* RS

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(b)

25

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GSTe2/RDL genotypes

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* SS/RS

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**Infected uninfected**

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