Insecticide resistance status of indoor and outdoor resting malaria vectors in a highland and lowland site in Western Kenya

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Abstract

Background: Long Lasting Insecticidal Nets (LLINs) and indoor residual spraying (IRS) represent powerful tools for controlling malaria vectors in sub-Saharan Africa. The success of these interventions relies on their capability to inhibit indoor feeding and resting of malaria mosquitoes. This study sought to understand the interaction of insecticide resistance with indoor and outdoor resting behavioral responses of malaria vectors from Western Kenya.

Methods: The status of insecticide resistance among indoor and outdoor resting anopheline mosquitoes was compared in *Anopheles* mosquitoes collected from Kisumu and Bungoma counties in Western Kenya. The level and intensity of resistance were measured using WHO-tube and CDC-bottle bioassays, respectively. The synergist piperonyl butoxide (PBO) was used to determine if metabolic activity (monooxygenase enzymes) explained the resistance observed. The mutations at the voltage-gated sodium channel (*Vgsc*) gene and *Ace 1* gene were characterized using PCR methods. Microplate assays were used to measure levels of detoxification enzymes if present.

Results: A total of 1094 samples were discriminated within *Anopheles gambiae s.l.* and 289 within *An. funestus s.l.* In Kisian (Kisumu county), the dominant species was *Anopheles arabiensis* 75.2% (391/520) while in Kimaeti (Bungoma county) collections the dominant sibling species was *Anopheles gambiae s.s* 96.5% (554/574). The *An. funestus s.l* samples analysed were all *An. funestus s.s* from both sites. Pyrethroid resistance of *An.gambiae s.l* F1 progeny was observed in all sites. Lower mortality was observed against deltamethrin for the progeny of indoor resting mosquitoes compared to outdoor resting mosquitoes (Mortality rate: 37% vs 51%, P=0.044). The intensity assays showed moderate-intensity resistance to deltamethrin in the progeny of mosquitoes collected from indoors and outdoors in both study sites. In Kisian, the frequency of vgsc-L1014S and vgsc-L1014F mutation was 0.14 and 0.19 respectively in indoor resting malaria mosquitoes while those of the outdoor resting mosquitoes were 0.12 and 0.12 respectively. The *ace 1* mutation was present in higher frequency in the F1 of mosquitoes resting indoors (0.23) compared to those of mosquitoes resting outdoors (0.12). In Kimaeti, the frequencies of vgsc-L1014S and vgsc-L1014F were 0.75 and 0.05 respectively for the F1 of mosquitoes collected indoors whereas those of outdoor resting ones were 0.67 and 0.03 respectively. The *ace 1* G119S mutation was present in progeny of mosquitoes from Kimaeti resting indoors (0.05) whereas it was absent in those resting outdoors. Monooxygenase activity was elevated by 1.83 folds in Kisian and by 1.33 folds in Kimaeti for mosquitoes resting indoors than those resting outdoors respectively.

Conclusion: The study recorded high phenotypic, metabolic and genotypic insecticide resistance in indoor resting populations of malaria vectors compared to their outdoor resting counterparts. The indication of moderate resistance intensity for the indoor resting mosquitoes is alarming as it could have an operational impact on the efficacy of the existing pyrethroid based vector control tools. The use of synergist (PBO) in LLINs may be a better alternative for widespread use in these regions recording high insecticide resistance.

Keywords: *Anopheles gambiae*, *Anopheles arabiensis*, *Anopheles funestus*, insecticide resistance, Indoor and outdoor resting behavior, Western Kenya

Introduction

Decline in malaria incidence and prevalence have been achieved in sub-Saharan Africa through the widespread use of anti-malarial drug therapies and scaling up of vector control interventions that primary target malaria vectors feeding and resting indoor [1]. Despite the observed improvement in malaria incidence and prevalence in many parts of sub-Saharan Africa, transmission is increasing in several countries [2,3]. The ongoing transmission has been partly attributed to the shifts in mosquito behaviours (biting and resting) due to increasing insecticide use for vector control [4-7] and increased insecticide resistance in the mosquitoes [8-10]. Malaria transmission is dependent on the propensity of malaria vectors to feed on human host and preference to live in close proximity to human dwellings [7].

Insecticide resistance in malaria mosquitoes is linked to presence and increase in metabolic detoxification enzymes, target site insensitivity and behavioural resistance [11]. Metabolic enzyme detoxification [12] and target site insensitivity [13] are responsible for higher levels of insecticide resistance [14]. Mechanisms that decrease the insecticide toxicity rely on modifications in one or several inheritable genes of the mosquito [11]. Detoxification enzyme systems that have been reported to confer resistance include three major families of enzymes; the cytochrome P450 monooxygenases, β-esterases, and the Glutathione S-transferases. In Western Kenya, about 80% of reported resistance genotypes are Vgsc-1014S *kdr* mutation, Vgsc-1014F mutations in the major vectors *Anopheles gambiae s.l.* (*An. gambiae* henceforth) and *Anopheles arabiensis* [15-18]. The malaria vector *Anopheles arabiensis* has been reported with increasing levels of *kdr* mutations [19]. There are no reports of *kdr* mutation at the locus 1014 in *Anopheles funestus*, also an important vector in Western Kenya and many parts of Africa despite having several reports of metabolic resistance [20-22]. The increasing levels of insecticide resistance in malaria mosquitoes

have been associated with continuous exposure to insecticides in Long Lasting Insecticide Nets(LLINs) [23,24] and agro-chemicals such as pesticides due to the creation of selection pressures [25-27].

Environmental changes have been implicated in the observed vector behavioural modifications, as mosquitoes could quickly adapt and respond by producing better matching phenotypes to prevent or reduce the negative consequences in the new environment [7]. For instance, field studies in East Africa have reported increased zoophagy [23,24,28], feeding outdoors or early evening indoors [29] and change in resting behaviour either indoor or outdoor [28,30,31]. These behavioural changes might have been due to selection pressure from increased coverage of LLINs [32-35]. The scale-up of LLINs in Africa has been associated with a species shift from the highly endophilic *An. gambiae* to the more exophilic *An. arabiensis* in Kenya [3,36,37]. The intervention pressure may selectively eliminate the most susceptible species from a population leaving the less vulnerable species able to adapt to the new environment [38]. While these field studies demonstrate the influence of environmental changes on the bahaviour of malaria vectors. Very little is known about the association of insecticide resistance and the behaviour of malaria vectors.

Given the importance of mosquito feeding and resting behaviour to the successes of malaria vector control and transmission, it is important to understand the influence of physiological resistance on the resting behaviour of malaria vectors and how the observed behaviours could impact the effectiveness of the existing frontline interventions. Currently, the mechanisms underlying the observed behavioural shifts in malaria vectors are poorly known, and it may have an epidemiological consequence. In order to maintain the efficacy of insecticide-based vector control, insecticide resistance must be constantly monitored and management strategies developed and deployed [8,39-43]. The present study attempts to answer how insecticide use and resistance

116 influences resting behaviours by reporting on the status of insecticide resistance in indoor resting
117 and outdoor resting malaria vectors.

118 **Methods**

119 **Study sites**

120 The study was carried out in the lowland site of Kisian (0.0749° S, 34.6663° E, 1,137m) in Kisumu
121 county and the highland site of Kimaeti (0.6029° N, 34.4073° E, 1,430m) in Bungoma county all
122 in Western Kenya (Fig. 1). These sites have high abundance of malaria mosquitoes (*An. gambiae*
123 *s.l.* and *An. funestus s.l.*) and high level of insecticide resistance [15,17]. Kimaeti (Bungoma
124 county) has extensive tobacco cultivation visible by large farms with numerous curing kilns
125 observed in the region. In Kisian (Kisumu county), there is sand harvesting from river beds,
126 fishing, rice and maize farming most of which enhance mosquito breeding habitats. There is
127 extensive use of agrochemicals on these farms which could have a potential role in the mediation
128 of resistance to insecticides. Western Kenya experiences long rainy seasons between the months
129 of March to June and the short rainy seasons between the months of October and November [44].

130 **Mosquito Sampling**

131 Resting *Anopheles* mosquitoes were sampled indoors and outdoors from household units.
132 Mosquito collections were made during the long rainy season (May-July) and the short rainy
133 season (October-November) of 2019. Prokopack and mouth aspirators were employed to collect
134 mosquitoes indoors. Outdoor collections were sampled from pit shelters dug (1.5M×1.5M×1.5M)
135 in the ground [45], from clay pots or containers placed at least 10 meters outside of houses and
136 from any proximal human outdoor resting points such as cowsheds and under shaded places.
137 Sampled anophelines were first discriminated using morphological keys [46]. Further species-

specific identification within the *An. gambiae s.l.* and *An. funestus s.l.* was conducted using PCR. Mosquito collections were done at the beginning and at the end of the dry and rainy seasons. This was done between 0600hrs and 1000 hrs. The samples collected were taken to the entomology laboratories at the Kenya Medical Research Institute (KEMRI), Center for Global Health Research (CGHR) for subsequent rearing, phenotypic, biochemical and molecular analyses.

Rearing of mosquitoes

Blood-fed and gravid female *Anopheles* mosquitoes from both the indoor and outdoor collections were aspirated into separate labeled netted mosquito holding cages measuring 30cm × 30cm × 30cm where they were maintained at 25 ± 2°C and relative humidity of 80 ± 4% with 12:12 hours of light and dark. They were provided with 10% sucrose solution imbibed in cotton wool. Oviposition cups were introduced into the cages for egg collection. Since all collections made were put together in similar cages, the number of mosquitoes that laid eggs was not determined. Eggs collected were transferred into larval rearing trays containing spring water where they hatched. The aquatic larval stages were maintained in water 26-27°C and were fed on a mixture of Tetramin™ fish food and brewer's yeast. After the four larval stages, pupae were picked and transferred into netted holding cages in small cups where the emergent adults were provided with 10% sucrose solution [47].

Testing phenotypic resistance in the F1 progeny of indoor and outdoor resting mosquitoes

First filial generation (F1) females raised from field-collected adults that were resting either indoors or outdoors, that were 3-5 -day old, were tested for susceptibility using the standard WHO tube bioassays (WHO, 2016) against discriminating doses of five insecticides selected from three classes: (i) Pyrethroids - (0.05% deltamethrin, 0.75% permethrin and (0.05% Alphacypermethrin);

and (ii) organophosphate - (5% malathion). For each test about 100-150 mosquitoes were used for the assay comprising 20-25 mosquitoes for each of four replicates for each of the insecticides and controls. Silicone oil-treated papers were used as a control for pyrethroid assays while olive oil was used for the malathion (organophosphate) test. Mosquitoes were exposed for 1hour for each insecticide and the number that were knocked down recorded after every 10 mins within the 1-hour exposure period. After 1-hour exposure to the diagnostic concentrations, mosquitoes were transferred to recovery cups and maintained on 10% sucrose solution for 24 hrs. Mortality was defined as the inability of the mosquitoes to stand or to fly in a coordinated manner. Mosquito survival status was examined at 24-hour post-exposure, where the survived and dead mosquitoes were collected and preserved at -20^{o}C prior to molecular analysis. Percentage mortality was calculated for both indoor and outdoor F1 mosquitoes.

Piperonyl butoxide (PBO) synergist bioassays

The involvement of oxidase (P450) resistance mechanism in pyrethroid resistance was determined by pre-exposing test populations to the oxidase inhibitor; Piperonyl butoxide synergist (PBO). Briefly, unfed females aged 3-5days were pre-exposed to 4% PBO impregnated test papers for one hour. After pre-exposure to PBO, the mosquitoes were immediately exposed to the three pyrethroids (deltamethrin, permethrin and alphacypermethrin) for an additional hour. One batch of 25 females was only exposed to 4% PBO without insecticide as a control. After pre-exposure to PBO and the insecticides, mosquitoes were transferred to holding tubes and supplied with 10% sugar solution. Mortality was recorded after 24 hours.

Measurement of insecticide resistance intensity in the F1 progeny

Insecticide resistance intensity testing to deltamethrin was determined by using CDC bottle bioassay with serial dosages. Serial concentrations (1×, 5× and 10×) of deltamethrin were prepared and used for the CDC bottle assays. The bottles were coated in batches for each working concentration, to which mosquitoes were exposed as per the CDC procedure guide MR4 [47,48]. The number of knocked-down mosquitoes was recorded every 10 minutes until either all mosquitoes in the test bottles were dead or it reached 1 hour after the start of the experiment. Mosquitoes were transferred to holding cups and fed on 10% sucrose solution. Mortality was recorded after 24-hours.

Molecular identification and genotyping of resistance alleles

Genomic DNA was extracted by the alcohol precipitation method and conventional PCR was used to speciate the samples [47,49,50]. The taqMan assay was used to detect the mutations (*Vgsc*-1014S, *Vgsc*-1014F and *N1575Y*) at the voltage-gated sodium channel [51,52] and the same set of samples were used to detect the *G119S* mutation in *Ace 1* [53].

Biochemical enzyme levels in F1 progeny of indoor and outdoor resting *An. gambiae s.l.*

From both sites, indoor and outdoor, 100-three-day old female mosquitoes, were killed by freezing for 10 minutes and homogenized individually in 0.1 M potassium Phosphate (KPO_4) buffer as described by Benedict, (2014). The levels of metabolic enzymes; β-esterases, Glutathione S-transferase (GST) and Oxidases were measured using microplate enzyme assays. To correct for variations in mosquito sizes, the protein content of each mosquito was measured by adding 20µl of mosquito homogenate to the microtiter plates in triplicates and 80µl of KPO_4 to each well after which 200µl of protein-dye reagent was toped up. A standard curve was used to relate amount of protein used. The absorbances were taken using a microplate reader [47,54,55].

Data analysis

The phenotypic resistance assays were expressed as proportions of mortality around 95% confidence interval and classified by WHO (2016) as a guide. Genotypic data for species identification was weighted as proportions of the samples assessed. The allele frequencies for resistant genotypes were calculated using the Hardy-Weinberg equilibrium equation. Metabolic resistance enzymes were analyzed by ANOVA after which the source of variation between the fold changes was determined by the Turkey-Kramer HSD test. All statistical analyses were done in R software version 3.6.3.

Ethical considerations

Scientific and ethical clearance was sought from the Kenya Medical Research Institute Scientific and Ethics Review Unit (SERU) under protocol number SERU 3616. The household heads and property owners were consulted and oral consent was obtained during indoor and outdoor mosquito sampling.

RESULTS

Species discrimination of *An. gambiae s.l.* and *An. funestus s.l.*

A total of 1094 samples were identified to species within the *An. gambiae s.l.* and 289 from the *An. funestus s.l.* from the two sites. In the lowland site of Kisian (Kisumu county), out of 520 *An. gambiae s.l.* samples analysed, *An. arabiensis* composition was 75.2% (95% CI; 71.5-78.9%) while *An. gambiae s.s.* was 24.8% (95% CI; 21.1-28.5%) . All 122 *An. funestus s.l* samples analysed from indoors were *An. funestus s.s.* (Table 1). In the highland site of Kimaeti (Bungoma county) out of 574 *An. gambiae s.s.* composition was 96.5% (95% CI; 95.0-98.0%) while *An. arabiensis* was 3.5% (95% CI; 2.0-5.0%). The 167 *An. funestus s.l.* analysed were all *An. funestus s.s.* (Table 1).

Phenotypic resistance in the F1 progeny of indoor and outdoor mosquitoes

A total of 2,800 female *An. gambiae s.l.* (Kisan=1,400 and Kimaeti=1,400) and 1,600 female *An. funestus s.l.* (Kisan=800 and Kimaeti=800) were used in the WHO tube assays. In the lowland site of Kisian, the mortality rate of the indoor resting *An. gambiae s.l.* mosquitoes exposed to deltamethrin was significantly lower than outdoors resting ones (37% ,95% [CI; 28-46%]) vs 51% [95% CI; 41-61%] respectively; t =2.035, df=6, P=0.044). The indoor resting *An. gambiae s.l.* had significantly lower mortality rate to permethrin than those resting outdoors (31% [95% CI; 22-40%] vs 51% [95% CI; 41-61%], t =2.078, df=6, P=0.042). Following exposure to alphacypermethrin, the mortality rate for indoor resting *An. gambiae s.l.* was 30% (95% CI; 21-39%) compared to their outdoor counterparts with 60% (95% CI; 50-70%) (t =4.392, df=6, P<0.05). There was 100% mortality for both the indoor resting and outdoor resting *Anopheles gambiae s.l.* when exposed to malathion. (Fig 2a)

Indoor resting F1 progeny raised from *Anopheles gambiae s.l.* collected from the highland site of Kimaeti had a mortality rate of 49% (95% CI; 39-59%) compared to those resting outdoors 53% (95% CI; 43-63%) when exposed to deltamethrin. Although the indoor resting mosquitoes showed a slightly lower mortality rate compared to outdoors, this was not statistically significant (t=0.474, df=6, P>0.05). Exposure of mosquitoes to permethrin showed for indoor resting mosquitoes had a significantly lower mortality 7% (95% CI; 1-12%) compared to those resting outdoors 51% (95% CI; 41-61%), (t =6.063, df=6, P<0.001). Mosquitoes exposed to alphacypermethrin on the other hand showed a mortality rate of 70% (95% CI; 61-79%) for indoor resting mosquitoes compared to those resting outdoors outdoors80% (95% CI; 72-88%), though this was not significantly different (t =1.058, df=6, P>0.05). Exposure of mosquitoes from the indoor or outdoor location in showed that *An gambiae s.l.* were fully susceptible to malathion with 100% mortality (Fig 2a).

Addition of PBO synergist to the test, partially restored the resistance of indoor resting mosquitoes from 37% to 96% for deltamethrin ($t=9.0$, $df=6$, $P<0.001$), 31% to 79% permethrin ($t=5.908$, $df=6$ $P=0.005$) and 30% to 92% for alphacypermethrin ($t=8.598$, $df=6$, $P<0.001$) in Kisian. The effects of the PBO synergist was evident in the outdoor resting mosquitoes with mortality rate range; 98%-100% for the three pyrethroids used, confirming the full involvement of monooxygenase enzyme activity in the pyrethroid detoxification (Fig 2a).

In Kimaeti, the addition of PBO to tests involving indoor resting *An. gambiae s.l.* showed significantly increased mortality rate from 49% to 100% ($t=7.095$, $df=6$, $P<0.001$) for deltamethrin, 7% to 95% ($t=16.436$, $df=6$, $P<0.001$) for permethrin and 70% to 99% ($t=5.385$, $df=6$, $P=0.001$) for alphacypermethrin. The effects of the PBO synergist was also seen in outdoor resting mosquitoes with the mortality rate ranging between 94% and 100% (Fig 2a).

Due to the small number collected outdoors and the general difficulty in raising the F1, only indoor *An. funestus s.l.* from both study sites were assayed. In Kisian, the mortality rate of *An. funestus* was 68% (95% CI; 59-77%) to deltamethrin, 74% (95% CI; 65-83%) to permethrin and 77% (95% CI; 69-85%) to alphacypermethrin (Fig 2b). In Kimaeti, the F1 of *An. funestus* showed mortality rates of 62% (95% CI; 52-72%) when exposed to deltamethrin, 89% (95% CI; 83-95%) to permethrin and 61% (95% CI; 51-71%) following alphacypermethrin exposure. There was 100% mortality across both sites with PBO pre-exposure (Fig. 2b)

Intensity of insecticide resistance in F1 of *An. gambiae s.l.* resting indoors and outdoors

The mortality rate for indoor *An. gambiae s.l.* from Kisian that were exposed to 1×, 5× and 10× of the diagnostic doses of deltamethrin was 42% (95% CI; 32-52%), 78% and 100% respectively whilst for outdoors was 51% (95% CI; 41-61%), 83% (95% CI; 76-90%) and 100%, indicating moderate-intensity resistance across both locations according to the WHO 2016 criteria [56] (Fig. 3**).** Although there was lower mortality among the indoor resting mosquitoes compared to their outdoor counterparts at 1× (t=1.269, df=6, P=0.130) and at 5× (t=0.823, df=6, P=0.221), this was not statistically significant (Fig 3).

The mortality rate of indoor resting population from Kimaeti exposed to 1×, 5× and 10× concentration of deltamethrin were 31% (95% CI; 22-40%), 75% (95% CI; 67-83%) and 100% respectively while the outdoors were 48% (95% CI; 38-58%), 80% (95% CI; 72-88%) and 100% respectively indicating moderate-intensity resistance in both locations according to the WHO 2016 criteria [56]. Similarly, even though the mortality rates were lower indoors than outdoors, there was no significant statistical difference between the two populations at 1× (t=1.512, df=6, P>0.05) and at 5× (t=0.808, df=6, P>0.05) (Fig. 3**).**

Target site genotyping for resistance alleles in the F1 of indoor and outdoor resting *An. gambiae s.l.*

In Kisian, the frequency of the vgsc L1014S and L1014F in the progeny of mosquitoes resting indoors were present with frequencies of 0.14 and 0.19 respectively for the F1of indoor resting mosquitoes whereas those raised from mosquitoes resting outdoors were 0.14 and 0.12 respectively. The *ace 1* mutation was present by higher frequency in the F1 of mosquitoes resting

289 indoors (0.23) compared to those of the ones resting outdoors (0.12). The vgsc-1014S and *ace 1*
290 mutations were not observed in *An. gambiae* from Kisian due to the small sample size.

291 The frequency of L1014S and L1014F present in mosquitoes collected indoors were 0.75 and 0.05
292 respectively in Kimaeti compared to those raised from mosquitoes collected outdoors (0.67 and
293 0.03 respectively). The ace 1 G119S mutation was obsereved in the F1 of mosquitoes resting
294 indoors with a frequency of 0.05 and was not present in those of mosquitoes resting outdoors. The
295 *kdr* point mutation at locus 1575Y was not present in both study sites (Table 2**).**

296

297 **Biochemical enzyme levels in F1 progeny of indoor and outdoor resting *An. gambiae s.l.***
298 The monooxygenases, β-Esterase and Glutathione S-transferases activities were analyzed to
299 determine the level of involvement in the F1 of *An. gambiae s.l.* insecticide resistance. In Kisian,
300 the monooxygenase activity was increased by 1.83 folds in the progeny of *An. gambiae s.l.* resting
301 indoors and by 1.66-folds for those resting outdoors when compared to the insectary reference
302 Kisumu strain ($F_{2,134}$=105.20, P<0.05, Fig. 4a). The β-Esterases fold change was not significantly
303 different between F1 progeny raised from indoor and outdoor resting *An. gambiae s.l.* mosquitoes
304 ($F_{2,134}$=188.50, P<0.05, Fig. 4b). In Kisian, the elevation of GSTs was by a 2.3-fold change in the
305 F1 of indoor-resting mosquitoes which was significantly higher than that of the F1 of those resting
306 outdoors ($F_{2,134}$=95.14, P<0.05, Fig. 4c).

307 The enzyme activity of monooxygenases was higher by 1.3-fold in the indoor population from
308 Kimaeti compared to the outdoor population ($F_{2,134}$=51.43, P<0.05, Fig 4a). The activity of β-
309 esterases from Kimaeti was elevated by 1.2 folds for the indoor-resting population which was
310 significantly different compared to that of the outdoor resting mosquitoes ($F_{2,134}$=36.66, P<0.001,

311 Fig. 4b). The activity of Glutathione S-transferase was elevated by a 3.0-fold change in the progeny
312 of mosquitoes found resting indoors than those found resting outdoors ($F_{2,134}$=119.9, P<0.05). (Fig.
313 4c).

DISCUSSION

This study set out to determine the level of insecticide resistance of *Anopheles* mosquito species between populations found resting indoors and those resting outdoors. Generally, high phenotypic, physiological (genotypic and metabolic) resistance was observed in the progeny of indoor resting malaria mosquitoes than the outdoor resting vectors.

In the lowland sites of Kisian (Kisumu county), *An. arabiensis* was the most abundant malaria vector compared to its sibling species *An. gambiae s.s.* whereas in Kimaeti (Bungoma county), the dominant species was *An. gambiae s.s*. similar to earlier reports [17,21,28,57,58]. The lowlands tend to have high temperatures and low humidity which favour the more resilient *An arabiensis* whereas in the highlands, there are low temperatures and high relative humidity which favour *An gambiae* [59].

The indoor population recorded high phenotypic resistance to pyrethroids than outdoors. The phenotypic insecticide resistance to pyrethroids in *An. gambiae s.l.* is widespread in Western Kenya evident in previous studies [15,17,19]. The resistance to pyrethroids by *An. funestus* was observed and has as well been reported before [60]. These regions of Western Kenya have been reported to have increasing resistance to pyrethroids which are the public health approved insecticides for use in LLINs [15,17,20,42]. There was 100% susceptibility to malathion of mosquitoes just as similar studies have shown in Ghana [61]. Synergist PBO pre-exposure restored susceptibility for both indoor and outdoor resting mosquitoes, revealing the role of detoxifying metabolic enzymes in the insecticide resistance in these regions. This means, therefore, that there are more factors at play contributing to the insecticide resistance present in Western Kenya similar to studies before [12,62,63]. Increasing the concentration of the deltamethrin in CDC bottle assays restored susceptibility to 100% suggesting that the continuous exposure to the current dosage in

337 LLINs and possible interaction with non-lethal doses in agricultural chemicals could have been at
338 play to contribute to the development of resistance to pyrethroids as previously demonstrated [38]
339 in indoor resting and outdoor resting malaria mosquitoes. The result showed moderate intensity
340 insecticide resistance since the mosquitoes succumbed to the highest concentration according to
341 the WHO test procedures for insecticide resistance monitoring in malaria vectors [56]. The buildup
342 of the phenotypic resistance which was higher in indoor resting mosquitoes compared to the
343 outdoor resting counterparts might be threatening current insecticide-based malaria control
344 interventions as suggested by prior studies [64,65].

345 The presence of resistance-associated point mutations was more in indoor resting mosquitoes than
346 their outdoor resting counterparts. This can be attributed to the adaptations from selection
347 pressures due to constant exposure to insecticide-based interventions such as LLINs [17,23,39,66]
348 and the extensive chemicals used in the tobacco farms in Kimaeti. The study also detected, even
349 though in lower frequencies, a significant proportion of the vgsc-1014S and 1014F in *An.*
350 *arabiensis* a phenomenon that has been previously reported [17,19,63]. This is in line with studies
351 that have shown the occurrence of more than one *kdr* associated point mutation within a population
352 of *An. gambiae s.l.* already reported previously [17,20,58,63,67]. The significant vgsc mutations
353 observed could be a result of selection pressure build-up that is due to more contact with
354 insecticides in indoor-based interventions [17,39,42,58,63]. From Kisian, the *G119S* mutation was
355 present at low frequencies even though it was higher in the progeny of mosquitoes resting indoors
356 compared to those resting outdoors. This was more in Kisian, where the vgsc mutations were at
357 lower frequencies than in Kimaeti. These findings suggest that these mutations could be arising
358 from different pressures that could be present in the lowland and absent in the highland.

359 The metabolic enzymes, associated with insecticide resistance (monooxygenases, β-esterases, and
360 glutathione S-transferases) activities were found to be elevated, more in indoor resting malaria
361 mosquitoes compared to the outdoor counterparts from both sites. From the phenotypic assays,
362 pre-exposure to PBO synergist restored the susceptibility of the malaria vectors to the pyrethroids
363 commonly used in LLINs by public health. Phenotypic exposures with prior PBO contact
364 demonstrated more activity of monooxygenases in aiding metabolic resistance. The involvement
365 of monooxygenases in pyrethroid resistance has been reported in Western Kenya [17]. In Kimaeti,
366 there was increased levels β-esterases, higher indoors than outdoors. Kisian, on the other hand, did
367 not show involvement of β-esterases in contributing to resistance as shown by similar levels in
368 indoor and outdoor resting mosquitoes. The glutathione-S-transferase possibly played a part in the
369 resistance levels as a previous study reported [68] since it was higher in mosquitoes resting indoors
370 than those resting outdoors from both Kisian and Kimaeti. These levels, therefore, suggest that
371 monooxygenases were the main mechanism of insecticide resistance in Kisian, especially with the
372 low frequency of resistant alleles, whereas in Kimaeti, the case pointed be a combination of
373 genotypic and metabolic mechanisms.

374 The expression of phenotypic, genotypic and metabolic resistance appears to be higher in indoor
375 than outdoor resting malaria mosquitoes in these regions. The widespread use of LLINs in attempts
376 to controlling these vectors and the extensive agrochemical use could be strengthening the increase
377 of insecticide resistance in the sites [21,58]. The higher levels indoors suggest that these
378 mosquitoes could be resting indoors because they are adequately resistant to the insecticides used
379 in LLINs, posing a threat to the wide coverage LLINs [21]. On the other hand, outdoors, the
380 resistance mechanisms were present as well pointing to exposure to these insecticide-based
381 interventions in just enough pressure to elicit expression of the resistance traits. The levels of

382 resistance could be enough to elicit an increase in malaria incidence due to the reduced mortality
383 of resistant malaria vectors that could hinder current vector control interventions [64].

384 **Conclusion**

385 In this study there was high phenotypic, genotypic and metabolic insecticide resistance in indoor
386 resting malaria vectors (*An. gambiae s.l* and *An. funestus*) compared to outdoor-resting
387 mosquitoes. Indoor-based insecticide control interventions are potentially at the verge of becoming
388 obsolete due to the reduced efficacy in controlling resistant malaria vectors which in turn might
389 lead to rise in malaria incidence. This calls for urgent improvement of these interventions and
390 development of alternative tools for indoor malaria control coupled with strengthening of
391 insecticide resistance monitoring. The use of synergist (PBO) in LLINs may be a better alternative
392 for widespread use in these regions recording high insecticide resistance.

393

394 **Authors' contribution**

395 KOO, MM, WRM, EO, GY and YAA designed the study and drafted the final manuscript. KOO,
396 MM participated in data collection, performed laboratory work, analysed data and drafted the
397 initial manuscript. WRM, EO, GY and YAA, supervised data collection. All authors have read and
398 approved the final manuscript.

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406

407 **Ethics approval and consent to participate**

408 Ethical approval for the study was obtained from Ethical Review Board of Kenya Medical
409 Research Institute under number SERU 3613. Permission was sought from community leaders of
410 each study site. Informed consent was obtained from the household heads. For mosquito larvae
411 collection, oral consent was obtained from field owners in each location. These locations were not
412 protected land, and the field studies did not involve endangered or protected species.

413 **Competing interest**

414 The authors declare that they have no competing interests.

415 **Availability of data and materials**

416 All relevant data are within the paper and its supporting information files

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599 arabiensis of different geographic origin. 5: 113.

600

601 **Table 1**: Number and percentage (in brackets) of *An. gambiae s.l.* and *An. funestus s.l.* species
602 composition from indoor and outdoor resting mosquitoes from Western Kenya

		***An. gambiae s.l.* (%)**		***An. funestus s.l.***	
Site	**location**	***An.gambiae s.s***	***An. arabiensis***	***An. funestus s.s.***	**Total**
Kisian	Indoor	83 (30.7)	167 (69.3)	122 (19.0)	392
	Outdoor	46 (18.4)	204 (81.6)	0	250
	Total	129 (24.8)	391 (75.2)	122	642
Kimaeti	Indoor	304 (99.02)	3 (0.97)	167 (23.16)	474
	Outdoor	250 (93.6)	17 (6.4)	0	267
	Total	554 (96.5)	20 (3.5)	167 (23.16)	721

603

605 **Table 2:** Frequency of resistant alleles (*Kdr* and *Ace1-G119S*) in indoor and outdoor-resting *An.*
606 *gambiae s.s* and *An. arabiensis* populations from Western Kenya

Site	Location	Species	n	Vgsc (*kdr*) Locus 1014 L1014S	Vgsc (*kdr*) Locus 1014 L1014F	Vgsc (*kdr*) Locus 1575 1575Y	Ace 1 Locus 119 G119S
Kisian	Indoor	*An.gambiae*	8	0	0.25	0	0
		An.arabiensis	36	0.14	0.19	0	0.23
	Outdoor	*An.gambiae*	1	0	0	0	0
		An.arabiensis	43	0.14	0.12	0	0.12
	Total	*An. gambiae*	9	0	0.33	0	0
		An.arabiensis	79	0.08	0.06	0	0.19
Kimaeti	Indoor	*An.gambiae*	43	0.75	0.05	0	0.05
		An.arabiensis	1	0.01	0	0	0
	Outdoor	*An.gambiae*	39	0.67	0.03	0	0
		An.arabiensis	5	0.60	0	0	0
	Total	*An.gambiae*	82	0.72	0.06	0	0.02
		An.arabiensis	6	0.07	0	0	0

607

608

609 **List of figures**

626

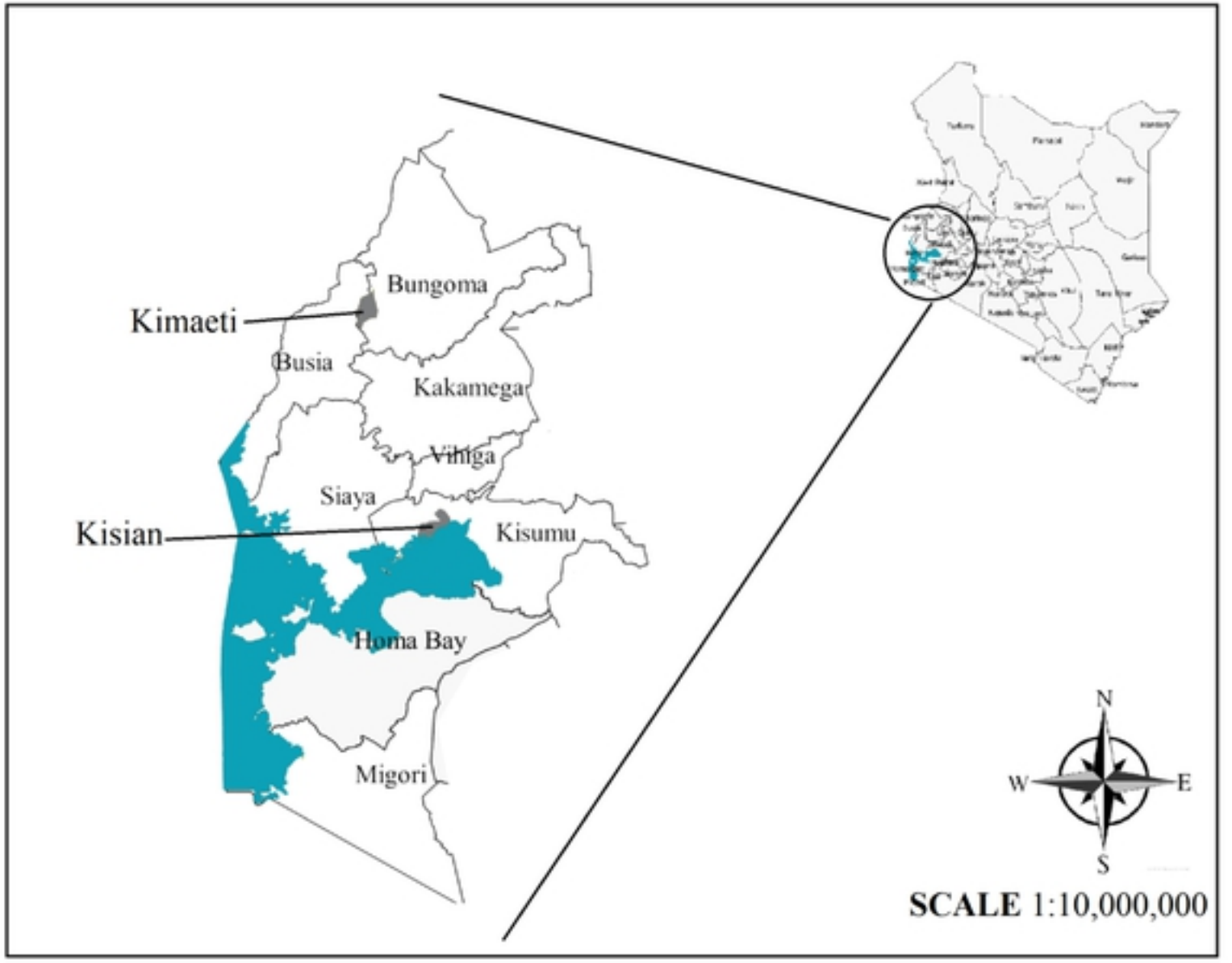
Bungoma
Kimaeti
Busia
Kakamega
Vihiga
Siaya
Kisian
Kisumu
Homa Bay
Migori
N
W
E
S
SCALE 1:10,000,000

Figure 1

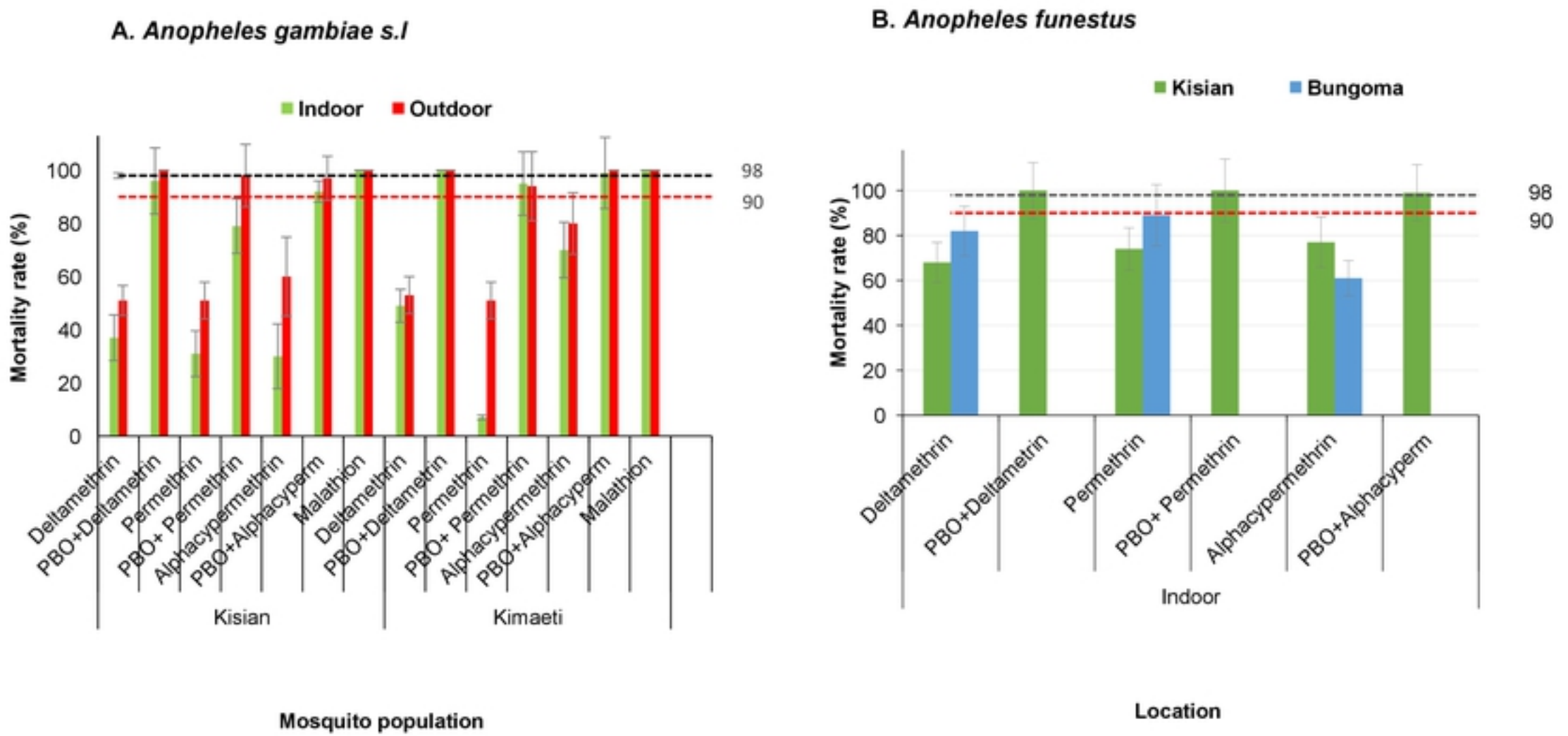
A. Anopheles gambiae s.l
Indoor
Outdoor
Mortality rate (%)
100
80
60
40
20
0
98
90
Deltamethrin
PBO+Deltamethrin
Permethrin
PBO+ Permethrin
Alphacypermethrin
PBO+Alphacyperm
Malathion
Deltamethrin
PBO+Deltamethrin
Permethrin
PBO+ Permethrin
Alphacypermethrin
PBO+Alphacyperm
Malathion
Kisian
Kimaeti
Mosquito population
B. Anopheles funestus
Kisian
Bungoma
Mortality rate (%)
100
80
60
40
20
0
98
90
Deltamethrin
PBO+Deltametrin
Permethrin
PBO+ Permethrin
Alphacypermethrin
PBO+Alphacyperm
Indoor
Location

Figure 2

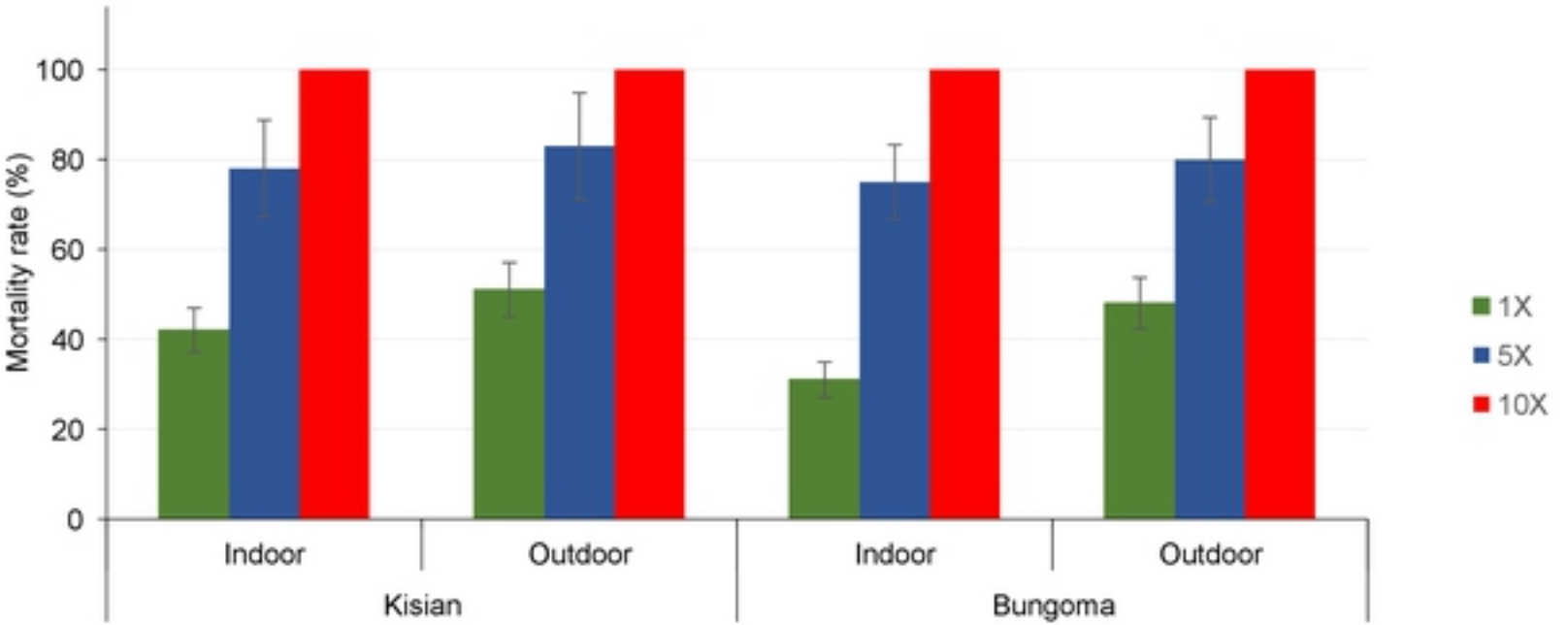
Mortality rate (%)
100
80
60
40
20
0
Indoor
Outdoor
Indoor
Outdoor
Kisian
Bungoma
1X
5X
10X

Figure 3

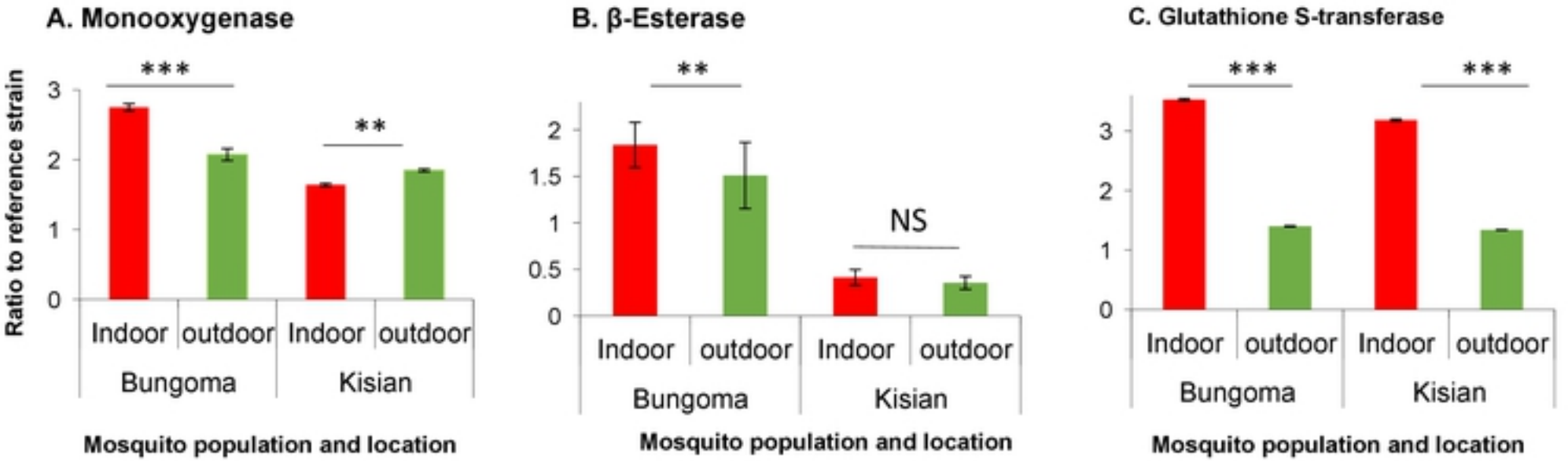
A. Monooxygenase
Ratio to reference strain
***
**
3
2
1
0
Indoor
outdoor
Indoor
outdoor
Bungoma
Kisian
Mosquito population and location
B. β-Esterase
**
NS
2
1.5
1
0.5
0
Indoor
outdoor
Indoor
outdoor
Bungoma
Kisian
Mosquito population and location
C. Glutathione S-transferase
***
***
3
2
1
0
Indoor
outdoor
Indoor
outdoor
Bungoma
Kisian
Mosquito population and location

Figure 4