RESEARCH ARTICLE

REVISED Pyrethroid resistance and gene expression profile of a new resistant *An. gambiae* colony from Uganda reveals multiple resistance mechanisms and overexpression of Glutathione-S-Transferases linked to survival of PBO-pyrethroid combination [version 2; peer review: 2 approved]

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v2 **First published:** 08 Jan 2024, **9**:13
https://doi.org/10.12688/wellcomeopenres.19404.1
Latest published: 29 Apr 2024, **9**:13
https://doi.org/10.12688/wellcomeopenres.19404.2

Abstract

Background

The effectiveness of long-lasting insecticidal nets (LLINs) are being threatened by growing resistance to pyrethroids. To restore their efficacy, a synergist, piperonyl butoxide (PBO) which inhibits cytochrome P450s has been incorporated into pyrethroid treated nets. A trial of PBO-LLINs was conducted in Uganda from 2017 and we attempted to characterize mechanisms of resistance that could impact intervention efficacy.

Methods

We established an *Anopheles gambiae* s.s colony in 2018 using female mosquitoes collected from Busia district in eastern Uganda. We first assessed the phenotypic resistance profile of this colony using WHO tube and net assays using a deltamethrin dose-response approach. The Busia colony was screened for known resistance markers and RT-qPCR targeting 15 genes previously associated with insecticide resistance was performed.

Open Peer Review

Approval Status ✓ ✓

	1	2
version 2 (revision) 29 Apr 2024	✓ view	✓ view
version 1 08 Jan 2024	? view	? view

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

Results

The Busia colony had very high resistance to deltamethrin, permethrin and DDT. In addition, the colony had moderate resistance to alpha-cypermethrin and lambda-cyhalothrin but were fully susceptible to bendiocarb and fenitrothion. Exposure to PBO in combination with permethrin and deltamethrin resulted in higher mortality rates in both net and tube assays, with a higher mortality observed in net assays than tube assays. The *kdr* marker, *Vgsc-995S* was at very high frequency (91.7-98.9%) whilst the metabolic markers *Coeae1d* and *Cyp4j5-L43F* were at very low (1.3% - 11.5%) and moderate (39.5% - 44.7%) frequencies respectively. Our analysis showed that gene expression pattern in mosquitoes exposed to deltamethrin, permethrin or DDT only were similar in comparison to the susceptible strain and there was significant overexpression of cytochrome P450s, glutathione-s-transferases (GSTs) and carboxyl esterases (COEs). However, mosquitoes exposed to both PBO and pyrethroid strikingly and significantly only overexpressed closely related GSTs compared to unexposed mosquitoes while major cytochrome P450s were underexpressed.

Conclusions

The high levels of pyrethroid resistance observed in Busia appears associated with a wide range of metabolic gene families.

Keywords
Uganda, Mosquito colony, Anopheles gambiae, Pyrethroids, Insecticide resistance, Long-lasting insecticidal nets (LLINs), Piperonyl butoxide (PBO), Bioassays, Gene expression, Glutathione-s-transferase (GST), Cytochrome P450s, Vector control

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Author roles: Oruni A: Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Lynd A**: Investigation, Supervision, Writing – Review & Editing; **Njoroge H**: Investigation, Writing – Review & Editing; **Onyige I**: Methodology; **van't Hof AE**: Investigation, Methodology, Writing – Review & Editing; **Matovu E**: Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing; **Donnelly MJ**: Conceptualization, Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Validation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by Wellcome [203511 https://doi.org/10.35802/203511], an International Master's Fellowship award to AO. Additional support was given by National Institute of Allergy and Infectious Diseases of the National Institutes of Health (R01AI116811) and a Royal Society Wolfson Fellowship (RSWF\FT\180003) awarded to MJD. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Oruni A, Lynd A, Njoroge H *et al.* **Pyrethroid resistance and gene expression profile of a new resistant *An. gambiae* colony from Uganda reveals multiple resistance mechanisms and overexpression of Glutathione-S-Transferases linked to survival of PBO-pyrethroid combination [version 2; peer review: 2 approved]** Wellcome Open Research 2024, **9**:13 https://doi.org/10.12688/wellcomeopenres.19404.2

First published: 08 Jan 2024, **9**:13 https://doi.org/10.12688/wellcomeopenres.19404.1

REVISED **Amendments from Version 1**

This second version addressed some of the concerns and corrections by the two reviewers. The reviewers wanted some clarifications on a number of items and this has been addressed in version two. For instance, clarifications on how colony was established, why selection was done at G12 with deltamethrin, WHO bioassays and qPCR experiment. The second version also contains a clearer Figure 1 and Figure 3. We also corrected two typos from *An. Gambaie* to *An. gambaie*.

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

Introduction

Uganda has one of highest malaria burdens in sub-Saharan Africa (WHO, 2018), with very high infection rates occurring in the eastern part of the country (Rugnao *et al.*, 2019). To control malaria, vector control efforts have been scaled-up over the past 20 years, mostly through the wide distribution of pyrethroid insecticide treated bed-nets (ITNs), supplemented by indoor residual spraying (IRS) (Katureebe *et al.*, 2016). However, the increase and spread in resistance to pyrethroids (Lynd *et al.*, 2018; Mawejje *et al.*, 2013a; Ranson & Lissenden, 2016), the primary insecticide used on bed-nets (WHO, 2016), is detrimental to the success of malaria control. Insecticide resistance is attributed to four main mechanisms: namely metabolic resistance, which occurs through increased expression or allelic variation in detoxifying genes that breakdown insecticides (Hemingway *et al.*, 2004); target-site resistance, which occurs through polymorphisms that alter insecticide binding sites (Donnelly *et al.*, 2016); as well as the less understood cuticular resistance and behavioral resistance. The high levels of pyrethroid resistance in Uganda (Mawejje *et al.*, 2013a; Mulamba *et al.*, 2014b; Okia *et al.*, 2018) and indeed the rest of sub-Saharan Africa (Lynd *et al.*, 2018; Ranson & Lissenden, 2016) is commonly associated with cytochrome P450 enzymes (David *et al.*, 2013; Djouaka *et al.*, 2016; Mulamba *et al.*, 2014b; Pondeville *et al.*, 2013; Scott, 1999; Wondji *et al.*, 2009). This has prompted the deployment of second-generation long-lasting insecticidal nets (LLINs) in which a synergist, piperonyl butoxide (PBO), is incorporated with the pyrethroid insecticide (WHO, 2017). The PBO acts by inhibiting the action of P450s, thus overcoming the metabolic resistance and restoring the killing effect of the pyrethroid (Bingham *et al.*, 2011; Snoeck *et al.*, 2017). Therefore, PBO-nets could help to partially check the resistance mediated by cytochrome P450s hence aiding effective vector control. Indeed, recently it was observed that children (2–10yr old) from communities with PBO-nets had lower malaria infection prevalence compared to children from communities with standard, non-PBO nets (Maiteki-Sebuguzi *et al.*, 2023; Staedke *et al.*, 2020). However, there has been an increasing trend in the intensity of metabolic resistance in several Anopheline mosquito vectors (Lynd *et al.*, 2018; Njoroge *et al.*, 2022; Stica *et al.*, 2019a; Weetman *et al.*, 2018). There are reports of mosquitoes surviving PBO-pyrethroid exposure from a number of countries in Africa including Uganda (Gleave *et al.*, 2021), but an understanding of the cause is lacking. Crucially, investigating the mechanisms by which mosquitoes overcome PBO exposure is vital for vector control and insecticide resistance management (IRM). There is already evidence for loss of efficacy to PBO nets against *An. funestus* attributed to both GSTs (Menze *et al.*, 2020) and P450 duplications (Mugenzi *et al.*, 2019) leading to successful blood-feeding by resistant mosquitoes.

In this study, we used a recently colonized *Anopheles gambiae s.s* line from Busia district in eastern Uganda to examine genetic variants potentially driving resistance with a focus on tolerance to PBO-pyrethroid combinations. The expression levels of 15 genes commonly associated with metabolic resistance in *An. gambiae* mosquitoes were investigated.

Methods

Ethics approval and consent to participate

The Wellcome Trust International Master's Fellowship (203511) project study was incorporated within the PRISM-study (NIH/NIAID U19AI089674) and the PBO-net study (R01AI116811) approved by the Ugandan National Council for Science and Technology (UNCST Ref HS 2176, on 20/12/2016), and the Liverpool School of Tropical Medicine (Ref 16-072, on 8/02/2017) which supported this study. Written informed consent to collect mosquitoes in the study was obtained from the head of household (or their designate) for all participating households.

Field mosquito collection and species diagnostic PCR

Mosquitoes were collected from 12 households in Busia District, Uganda, within the South-Bugwere Health sub-District (HSD) (0°19'01.0"N 33°58'00.5"E). Houses were selected from an ongoing cohort study based on data showing malaria cases reported within one year. From the top 12 houses on the list, household heads were approached, the aim of the study explained to them and their permission sought to collect mosquitoes from their houses. Once they consented, they were informed a day before the collection about the activities. Indoor-resting, blood-fed, female mosquitoes were collected using a Prokopack aspirator between 05:00 and 08:00 between 01st October and 19th November 2018. Mosquitoes were transported in a cool box to a nearby insectary and morphologically identified as *An. gambiae* s.l before being transferred into standard 30cm^3 BugDorm cages (Watkins & Doncaster, Leominster, UK). We used species diagnostic PCR (Chabi *et al.*, 2019) to differentiate *An. gambiae s.s.* from *An. arabiensis* collected from the field and at G1 generation to assess colony composition.

Establishment of the BusiaUG colony

Prior to collection of mosquitoes for colony establishment, a baseline collection was done and used to rear progeny (F1s) that was used to ascertain the resistance profile of the *Anopheles gambaie* population in Busia. The F1 mosquitoes were reared at Nagongera insectary in Tororo district under standard insectary conditions and used solely for bioassays. Once the resistance profile was ascertained, a total of 384 female mosquitoes were collected, of which 150 gravid females were made to oviposit by forced egg laying method. Briefly, a gravid female about 5 days old is placed inside a 1.5ml Eppendorf tube containing moist filter paper. Three holes are then pierced at the top of the lid. Mosquitoes are then kept for 24 to 48 hours

to allow oviposition (Morgan *et al.*, 2010). The remaining females laid eggs by cage oviposition where a moist Whatman filter placed on an oviposition cup is placed inside the cage to allow oviposition. The resulting eggs were kept at 4–10°C prior to and during shipment to Liverpool where they were immediately transferred to hatching containers on arrival. Mosquitoes were reared following standard insectary protocols. Emerged adults were kept in a large polyester mesh net measuring 60×60×60 cm to improve mating success. Mosquitoes were then subdivided into groups of approximately 200 mosquitoes each at day-5 and kept in ten smaller cages measuring 20×20×20 cm in which they were also blood fed. Female mosquitoes were arm fed in the dark following an overnight starvation of 8 hours, and then maintained on 10% sucrose. A decrease in oviposition rates was observed from generation one (G1) to generation six (G6) which we attempted to overcome by biweekly blood feeds and multiple feeding attempts per day. Adults from different oviposition rounds but the same generation were pooled into single cages. Females were provided with a black cup for oviposition. This procedure was repeated for about 20 weeks until the colony was stable and a higher number of emerged adult mosquitoes (>200 mosquitoes) were obtained from a single oviposition. For this study, mosquitoes from up to 11th generation (G11) which were unselected, were used. Insecticide resistance selection was performed at the 12th generation (G12) using 0.05% Deltamethrin to maintain pyrethroid resistance in the colony into the subsequent generations. The colony was named "BusiaUG" where "Busia" denoting the district where the mosquitoes were collected and "UG" denoting Uganda.

Mosquito rearing conditions

All *An. gambiae* s.l were reared under standard insectary conditions at temperature of between 25–28°C with 75–85% relative humidity under a 12:12 photoperiod. Two colonies (Tiassale and Kisumu) were used for comparative purposes. Tiassale was established in 2009 in Cote d'Ivoire and has been maintained under selection using pyrethroids at LSTM since 2013. It became resistant to pyrethroids and DDT by 2018 with only *Vgsc-995F kdr* genotype but low level of fenitrothion resistance with both the G119S and 119G *Ace-1* genotypes present. The Kisumu strain, from western Kenya, is susceptible to all pyrethroids and carbamates but exhibits some tolerance to DDT (Williams *et al.*, 2019).

WHO bioassays (resistance phenotyping)

For all resistance phenotyping, 3–5-day old females were used. In the baseline collection, reared F1 mosquitoes were exposed to four types of LLINs using the WHO cone assays for 3 minutes, after which 1hr knockdown and 24hr mortality were recorded, following WHO protocol (WHO/GMP, 2016). In addition, during the colony establishment, G1 mosquitoes were exposed to discriminating dose of deltamethrin (0.05% - 1x) using the WHO tube assay (WHO/GMP, 2016), and a concentration series (2x, 3x, 5x and 10x), to assess the level of resistance. Standard WHO impregnated deltamethrin papers were used for 1x, 5x, and 10x while the 2x and 3x were lab prepared using the WHO protocol. Furthermore, we couldn't test other insecticides on G1s because we wanted to maximize the number of mosquitoes for the colony establishment. After successfully establishing the colony, mosquitoes from G9–G11 were exposed to other types of insecticides using standard WHO insecticide impregnated papers which included; 0.05% deltamethrin, 0.75% permethrin, 4% DDT, 0.05% cypermethrin, 0.05% cyhalothrin, 1% fenitrothion, 0.1% bendiocarb and PBO (4%) synergistic assays with deltamethrin or permethrin. All procedures were conducted according to WHO guidelines (WHO/GMP, 2016). Mosquitoes were kept on 10% sucrose during the 24hr recovery period. Mosquitoes that survived insecticide exposure were kept at -80°C for RNA extraction, whilst dead mosquitoes were stored on silica gel for DNA extraction.

During exposure of mosquitoes to insecticides using WHO tube assays, a minimum of 2 (n=2) and maximum of 4 replicate experiments comprising of ≈25 alive mosquitoes were performed to obtain 5–10 pools of alive mosquitoes for each insecticide type for gene expression analysis. For deltamethrin, permethrin, DDT, PBO + deltamethrin, bendiocarb and fenitrothion, four replicates were used; for PBO + permethrin, three replicates) were used, and for alpha-cypermethrin and lambda-cyhalothrin, two replicates were used.

Resistance genotyping

DNA was extracted from the mosquitoes using the nexttec™ 1-Step kit (Biotechnologie GmbH, Hilgertshausen, Germany) according to manufacturer's instructions. Molecular identification was used to discriminate *An. gambiae* s.s. and *An. arabiensis* (Chabi *et al.*, 2019). Mosquitoes were genotyped for the common resistance markers; *Coeae1d*, *Cyp4j5* (Weetman *et al.*, 2018) and *Vgsc*-L995F/S (*Kdr*) (Lynd *et al.*, 2018).

RT-qPCR

To extract RNA, 5–10 mosquitoes were pooled to make a single biological replicate using only female mosquitoes which survived insecticide exposure (alive), for each treatment. The groups of alive mosquitoes after exposure to insecticides included; deltamethrin, permethrin, DDT, alpha-cypermethrin, lambda-cyhalothrin, PBO + permethrin, PBO + deltamethrin and PBO only (as experimental control) and unexposed mosquitoes from the BusiaUG colony, Kisumu (as susceptible control) and Tiassale (as resistant control). RNA was extracted from all the groups except Tiassale which was obtained from an earlier study (Ingham *et al.*, 2018). RNA was extracted using the PicoPure® RNA Isolation Kit, (ThermoFisher scientific, United Kingdom) followed by cDNA synthesis using the SuperScript® III First-Strand Synthesis System, ThermoFisher (United Kingdom) and subsequent purification of the cDNA using a QIAquick® PCR Purification Kit (Qiagen). All three procedures were done according to manufacturers' manual. Both RNA and cDNA were stored at -80°C.

Expression patterns of 15 potential metabolic resistance genes were investigated (Edi *et al.*, 2014; Ingham *et al.*, 2018; Irving *et al.*, 2012; Mulamba *et al.*, 2014a; Stica *et al.*, 2019b; Wilding *et al.*, 2014). Primers for qPCR were re-designed using the DNA sequences from the Ag1000g database (The Anopheles gambiae 1000 Genomes Consortium, 2017) to avoid mutations that could affect annealing (supplementary Table S1, Oruni, 2023d). Reactions were carried out in a final volume of 20 µl

consisting of 1x Brilliant III Utra-Fast SYBR® mix (Agilent Technologies, United Kingdom), 300nM of each primer and 1.0µl of 1:10 diluted cDNA. The qPCR assay was performed on the AriaMX Real-time PCR System (Agilent technologies) with an initial denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 10s, 60°C for 30s). A final melt curve from 55°C to 95°C was performed to check amplification quality. Standard curves for all primer sets were carried out using Kisumu cDNA to determine primer efficiency. Cycle threshold (Cq) values were analyzed using the ΔΔct Pfaffl method (Pfaffl, 1999) and comparative Ct method (Schmittgen & Livak, 2008) using the RPS7 gene as an endogenous control for normalization of expression. Gene expression of BusiaUG survivors for each insecticide exposure were compared to the susceptible population (Kisumu) and the fold change reported.

For the RNA experiments, three biological replicates (n=3) were used, except for lambda-cyhalothrin and PBO + deltamethrin exposed groups where two biological replicates (n=2) were used. The variation in number of biological replicates was because only 12 live mosquitoes were obtained (giving 6 mosquitoes per replicate) from PBO-deltamethrin exposure, while for lambda-cyhalothrin, one of the replicates had very low RNA concentration and was discarded. However, in all the RNA experiments, three technical replicates (n=3) were used. All primer pairs, except for the *Cyp4c28* gene (with efficiency of 76.3% and hence not discussed further) were within the acceptable efficiency range of 90–120%.

Gene expression analysis

We used Ct-values to calculate relative fold change. The resistant population comprised of the BusiaUG treatment groups (exposed, unexposed) and unexposed Tiassale, while the reference control susceptible population was the Kisumu laboratory strain. Using the unexposed group as the baseline, we calculated relative fold change expression for all exposed groups. Genes that were overexpressed and those that were downregulated in resistant population of *An. gambiae s.s* from eastern Uganda following exposure to pyrethroids or PBO were identified. The mean fold change of gene expression for all groups was visualised using heatmaply package on R software. ANOVA was used to calculate the significant differences in mean fold changes between the exposed and unexposed groups, with a threshold at P=0.05. To further investigate the gene expression pattern observed in mosquitoes exposed to PBO, a STRING network analysis (Szklarczyk *et al.*, 2019) was used to identify the relationship between the genes.

Results

An. gambaie s.l species composition

Of the 348 mosquitoes obtained in the first household collections for preliminary assessment of field resistance, 95% were *An. gambiae s.s* and 3.4% *An. Arabiensis* (Oruni, 2023a & Oruni, 2023b). A total of 384 mosquitoes were obtained in the second collection for colony establishment, of which 97.7% and 2.3% were *An. gambiae s.s* and *An. arabiensis* respectively. During colony establishment the collections were mixed, although all *An. arabiensis* were lost at G1 such that 100% of the G2 population were *An. gambiae s.s.*

Phenotypic resistance profile

Female mosquitoes collected from the field and BuisaUG colony at generation one (G1) and nine (G9) were exposed to an array of insecticides. Female mosquitoes exposed to only pyrethroids commonly used in LLINs i.e. deltamethrin and permethrin, or DDT showed very high resistance both in the field and from the colony. WHO cone assays were only done using G1 mosquitoes which showed very low mortality to pyrethroid-only nets; PermaNet 2.0 (6.0%, 95% CI; 5.6-6.4) and Olyset Net (4.0%, 95% CI; 3.8-4.2; 95%) (Figure 1A). WHO tube assays also caused low mortality with deltamethrin, permethrin and DDT impregnated papers (Figure 1C). In addition, exposure to 5x and 10x the discriminatory dose did not result in complete mortality (Figure 1B). Full susceptibility (100% mortality) was only observed with carbamates and organophosphates in WHO tube assays (Figure 1C).

Exposure of mosquitoes to PBO using either PBO-net cone assays, or pre-exposure in WHO tube assays significantly increased mortality to permethrin and deltamethrin, suggesting a role for cytochrome P450s in resistance. In cone assays against dual LLINs, full susceptibility was observed against the top surface of PermaNet 3.0 (deltamethrin + PBO), whilst 80.0% (95% CI; 76.1-83.9; 95% CI) mortality was observed against Olyset Plus (permethrin + PBO) (Figure 1A). A similar range of mortality was observed with WHO synergistic assays (Figure 1C).

Frequency of resistance markers

Markers previously associated with resistance in East Africa (Lynd *et al.*, 2018) were assessed in field collected mosquitoes and in G1 mosquitoes of the BusiaUG colony. All three genotypes of the mutant kdr alleles (serine (S), phenylalanine (F) and heterozygote (FS)) were present at the Vgsc-995 locus in the field population. The allele frequency of *Vgsc-995S* allele was 87.5% in *An. gambiae s.s.* and 4.2% in *An. arabiensis* and that of the *Vgsc-995F* was 3.13% all from *An. arabiensis* based on the sampled mosquitoes. All resistance genotypes for the metabolic marker *Cyp4j5-L43F* and *Coeae1d* variant were confirmed present at 1.3%, 78.9% and 19.7% for mutant allele, heterozygote and wild-type allele for *Cyp4j5-43F* and 44.7%, 43.4% and 14.5% for *Coeae1d* (Table 1).

Transcriptomic (Gene expression) profiles

To examine differential gene expression, both exposed and unexposed mosquitoes were compared to susceptible mosquitoes, and we noted an upregulation of most genes from all the gene families studied with highest expression of GSTMS3 in permethrin survivors (mean fold change =27.98) (Figure 2) (supplementary figure S1, Oruni, 2023c). To further examine if these genes were under induced or constitutive expression, we compared the exposed Busia mosquitoes with unexposed mosquitoes and of the 15 genes examined, 14 genes were overexpressed in mosquitoes that were exposed to the insecticides used in LLINs, deltamethrin and permethrin (Figure 3A–D). All P450s showed evidence of induced expression although only CYP6P4 (14.19-17.49-fold), CYP6AA1 (11.16-11.44-fold), CYP6P3 (11.22-18.43-fold) and CYP6Z3 (6.4-7.05-fold) were significantly upregulated. The CYP4C28 gene was not considered because of low primer efficiency.

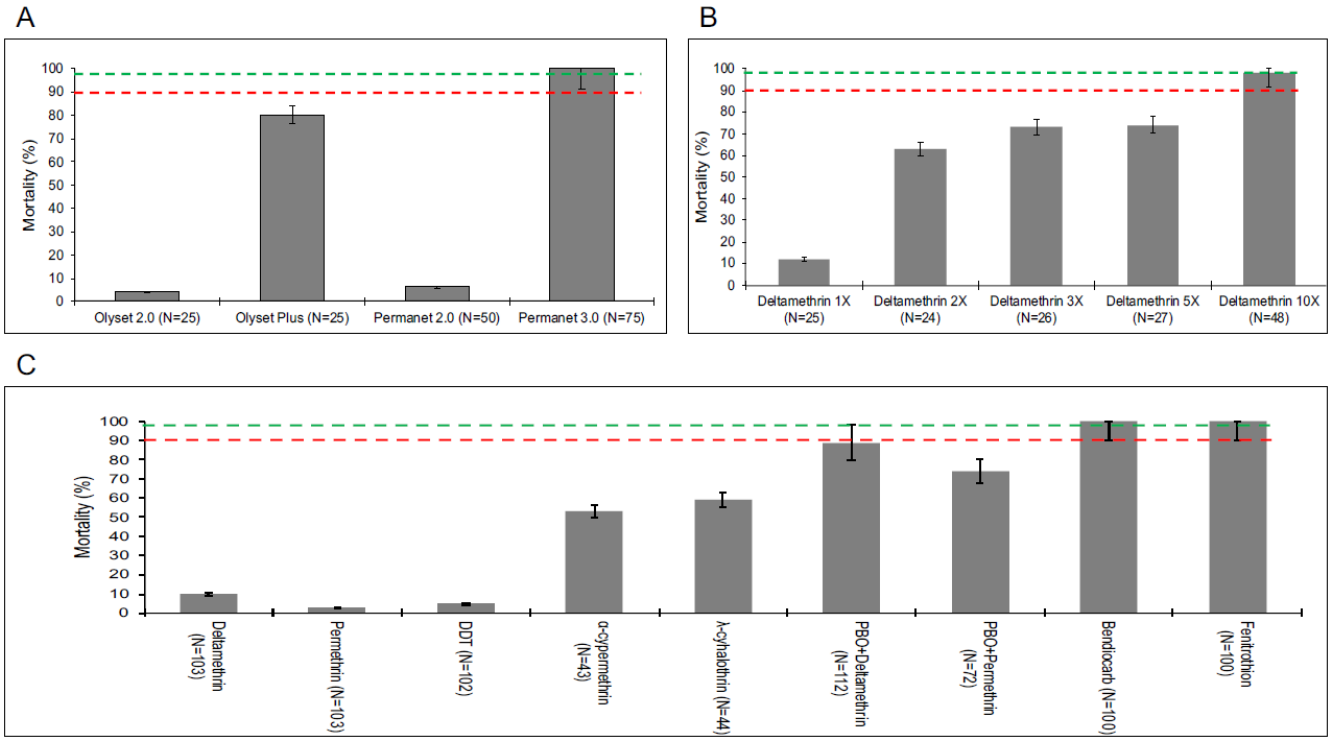

Figure 1. Resistance profile of *An. gambiae s.s* mosquitoes from the field and BusiaUG colony following exposure insecticides. Female mosquitoes from Busia exhibited very high resistance (low % mortality) when exposed to pyrethroids without piperonyl butoxide (PBO) by both field WHO net assays (permethrin-Olyset 2.0 or deltamethrin-PermaNet 3.0) (panel **A**) and WHO tube assays on the colony at G1 (panel **B**) or G9 (panel **C**). Colony female mosquitoes also showed high intensity of resistance by surviving deltamethrin at 5x and 10x with increased mortality at higher doses (**B**). alpha-cypermethrin and lambda-cyhalothrin showed moderate resistance and there was full susceptibility to organophosphates and carbamates (**C**). The mosquito mortality by pyrethroids greatly increased when females were exposed in presence of a cytochrome P450 inhibitor, PBO, both with the WHO net assays using Olyset Plus and PermaNet 3.0 top (**A**) and WHO synergistic tube assays (**C**). Higher mortality was observed with WHO net assays using PBO nets compared to WHO tube assays especially with PermaNet 3.0 but not with Olyset Plus. Dotted lines indicate resistance levels where above 98% (green) it's susceptible while below 90% (red) is confirmed resistance. Between 98% and 90% is suspected resistance. The error bars are SEM at 95% confidence interval.

Table 1. Allele frequency of resistance markers from *An. gambiae s.s* mosquitoes sampled from the field and BusiaUG colony. RR represents the homozygous allele for resistance, RS represents the heterozygous allele and SS represents the homozygous allele for susceptibility.

Test sample	Marker	N	Frequency		
			RR	RS	SS
G1 from the field	*Vgsc*-L995S	96	88(91.7%)	0 (0.0%)	0 (0.0%)
	Vgsc-L*995F*	96	3 (3.13%)	5(5.2%)	0 (0.0%)
	Cyp4j5-L43F	92	11 (11.5%)	58 (60.4%)	23 (24.0%)
	Coeae1d	95	38(39.5%)	32(33.3%)	25(26.0%)
G1 from the BusiaUG colony	*Vgsc*-L995S	90	89 (98.9%)	0 (0.0%)	1(1.1%)
	Vgsc-L995SF	90	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Cyp4j5-L43F	76	1 (1.3%)	60 (78.9%)	15 (19.7%)
	Coeae1d	78	34(44.7%)	33(43.4%)	11(14.5%)

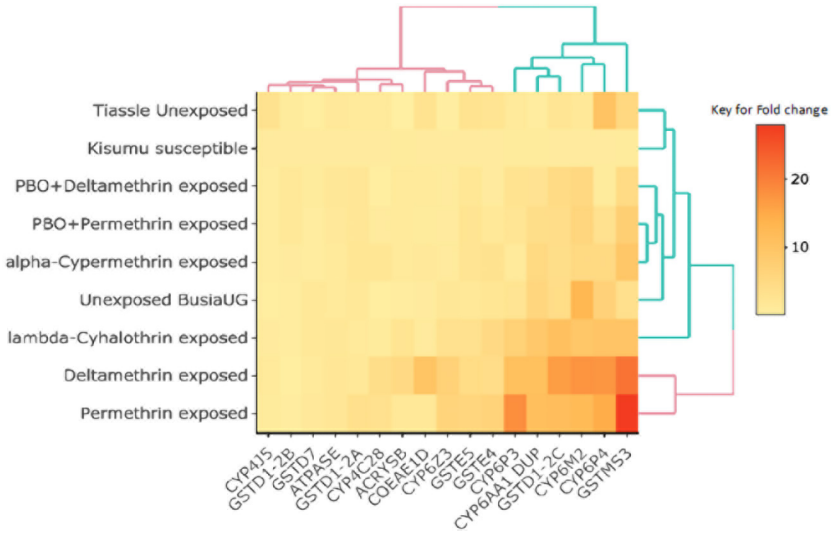

Figure 2. Heat map showing differential gene expression of the selected candidate genes from the different treatment groups relative to the susceptible control. The colours represent the fold change values with yellow being the lowest and red the highest. The heatmap was generated using R software (heatmaply package using k=2).

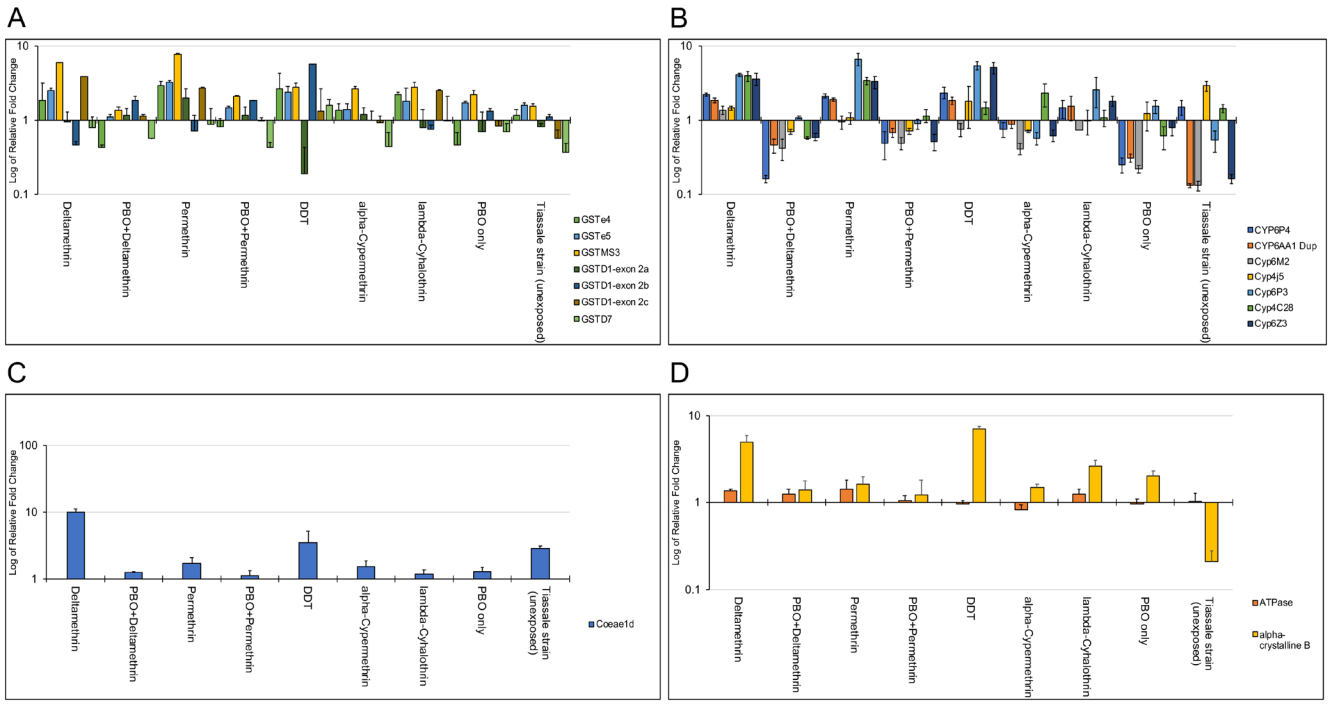

Figure 3. Relative fold change of gene expression of major gene families; GSTs, P450s and COEs, in treatment groups compared to unexposed Busia mosquitoes (resistant population). Transcription profiles of metabolic genes associated with insecticide resistance in Africa analysed by RT-qPCR shows unique expression patterns between mosquitoes exposed to PBO and non PBO. Mosquitoes pre-exposed to PBO only significantly overexpressed GSTMS3, GSTe5 and GSTD1 (**A**) but down-regulated of all major P450s (**B**). Mosquitoes exposed to only pyrethroids and DDT overexpressed almost all major genes including a COE, Coeae1d gene (**C**) and genes for ATPase and alpha-crystalline proteins (**D**). The west African resistant strain, Tiassale had a similar expression pattern for GSTs compared to BuisaUG colony though at lower levels but a different expression pattern for P450s. Data shows mean + SEM (error bars).

Within the GST gene family (except GSTD7), all the genes – GSTMS3 (21.54-27.98-fold), GSTE4 (4.23-6.63-fold), GSTE5 (4.73-6.05-fold), GSTD1-exon 2C (11.77-16.71-fold) – were significantly upregulated following exposure. The expression patterns of CYP6M2, COEAE1D and GSTMS3 were less consistent where there was no significant difference in overexpression of CYP6M2 in exposed and unexposed mosquitoes (ANOVA; *F-stat* = 1.589, P = 0.276), COEAE1D was only significantly upregulated (10.19-fold, ANOVA; *F-stat* = 45.2899, P = 0.0025) in deltamethrin survivors, but not in those surviving exposure to other insecticides including other type 2 pyrethroids, alpha-cypermethrin and lambda-cyhalothrin, although

exposure to alpha-cypermethrin and lambda-cyhalothrin caused a significant upregulation of only GSTMS3 (P<0.05). The GSTMS3 gene was significantly overexpressed in both non-PBO and PBO exposed mosquitoes. Strikingly, mosquitoes that were exposed to the synergist-PBO, showed that expression of GSTs was not significantly affected (ANOVA; F-statistic =2.13113, p=0.17001 for deltamethrin and ANOVA; F-statistic =2.11146, p=0.17185 for permethrin) compared to P450s where there was significant reduction in gene expression (ANOVA; F-statistic =10.30138, p=0.0075 for deltamethrin and ANOVA; F-statistic = 7.79701, p=0.01627 for permethrin) (supplementary Figure S1, Oruni, 2023c). When compared to unexposed BusiaUG, all P450s showed expression levels below the unexposed groups which was not seen in GSTs, COEs, ATPase and alpha-crystallin (Figure 3A–B). The GSTs overexpressed in PBO-exposed mosquitoes, GSTMS3 (4.91-7.91-fold), GSTE5 (2.05-2.72-fold) and GSTD1 (4.17-4.49-fold), were significant in one or both PBO-pyrethroid exposed groups. However, GSTD7 and GSTE4 seemed to have been under-expressed after mosquitoes were exposed to PBO, similar to what was observed with the P450s. To understand this expression pattern, A STRING network analysis of the significantly overexpressed GSTs revealed multiple interaction networks including co-expression and similarity in protein homology (supplementary figure S3, Oruni, 2023c). The STRING analysis looks at both direct and indirect protein-protein interaction determined from both experiments and databases. The resultant outcome reveals whether proteins interact through co-expressions, gene fusions, occurrence, or all of them. The interpretation of this is whether proteins interact or just have similar pathways during gene expression.

A comparison of expression profiles between the unexposed resistant strains of BusiaUG and the Tiassale colony derived from West Africa, exhibited distinct transcriptomic profiles (Figure 3C). Only seven out the 15 selected genes, that were all overexpressed in BusiaUG mosquitoes, were overexpressed in Tiassale and three out of the seven were significantly expressed, although at lower levels.

Discussion

We detected very high levels of resistance to pyrethroids and DDT in Busia, eastern Uganda, with *Vgsc* kdr resistance nearly at fixation and moderate levels of the metabolic markers, *Cyp4J5* and *Coeae1d*, as has been previously reported (Lynd *et al.*, 2019; Ojuka *et al.*, 2015). Resistance to the other type-2 pyrethroids, alpha-cypermethrin and lamda-cyhalothrin, which are not commonly used in Uganda, were less severe (50–60% mortality) compared to deltamethrin (<5% mortality). Although the suggested strategy would be recommending substitution of alpha-Cypermethrin or lambda-Cyhalothrin for permethrin in LLINs through the deployment of nets such as VEERALIN® and DuraNet®, recent evidence seems to suggest that this is not advisable as an IRM strategy since differences in susceptibility does not necessarily indicate operational relevance in performance unless the mode of action of the insecticide is completely different (Lissenden *et al.*, 2021). Carbamates and organophosphates are still fully effective against *An. gambiae* s.s. from Busia, and likely from eastern Uganda which is concordant with previous reports (Mawejje *et al.*, 2013b; Okia *et al.*, 2013). The continued use of IRS in addition to PBO-LLINs may benefit vector control programs in the region as has been previously reported (Katureebe *et al.*, 2016). The PBO-LLINs however, can only be used to complement IRS but not together. Recent evidence shows that combining IRS with PBO-LLINs can significantly reduce the effectiveness of the IRS with pirimiphos-methyl but not with bendiocarb in a resistant population of *An. gambiae* (Syme *et al.*, 2022). Nonetheless, although IRS programs coupled with LLINs may produce mixed results in some cases, usage of Actellic 300CS® (pirimiphos-methyl) has been quite effective in reducing malaria cases in Uganda (Epstein *et al.*, 2022). However, the intense resistance levels are of concern for the continued usage of conventional LLINs (LLINs without PBO) that may only help prevent bites but not deliver mosquito lethality, thus providing only personal protection rather than community benefits (Gleave *et al.*, 2021; Kleinschmidt *et al.*, 2015). Previous work showed that standard LLINs may not be sufficient to sustain malaria vector control even if supplemented by IRS using carbamates (Katureebe *et al.*, 2016). On a positive note though, our results showed that exposing mosquitoes to a synergist, piperonyl butoxide (PBO), significantly increases mosquito mortality by pyrethroids which has also been reported previously (Mawejje *et al.*, 2013b). Furthermore, we noted that deltamethrin PBO nets appear to be very effective against *An. gambiae* resistant populations and may therefore offer community protection. Our findings are in line with a recent cluster-randomized trial of PBO nets in Uganda, where malaria parasite prevalence in 2-10-year olds were significantly lower in areas where PBO nets were distributed compared to conventional LLINs after 18 months (Staedke *et al.*, 2020) and 25 months (17.7% parasite prevalence in PBO-LLINs compared to 19.6% in Non PBO-LLINs, p=0.005) (Maiteki-Sebuguzi *et al.*, 2023) post distribution. Maiteki-Sebuguzi *et al.* further showed that this reduction in malaria cases in PBO-LLINs areas might have been associated with reduction in vector density (542 mosquitoes collected in PBO-LLINs compared to 905 in Non PBO-LLINs, p<0.001). The effectiveness of PBO-LLINs in Uganda is reassuring, however, this does come with increased selection pressure from the mass distribution. It is possible that resistance to PBO nets could emerge and become widespread. In several studies reported by Gleave *et al.*, analysis of mosquito mortality by PBO-LLINs suggests that this could already be happening elsewhere since the nets have been in use more frequently in some regions (Gleave *et al.*, 2021). Using a time series analysis of the different studies, PBO and non PBO net efficacy was compared from experiments involving huts. In the analysis, studies were clustered into net type (PermaNet 3.0, Olyset Plus, PermaNet 2.0 and Olyset 2.0) and population resistance levels (high, moderate, low to susceptible). The results revealed the ability of PBO nets to kill mosquitoes decreased with time (from 2010 to 2018) for both PermaNet 3.0 and Olyset Plus in populations of *An. gambiae* mosquitoes that were highly resistant and only very effective in low to susceptible populations (supplementary figure S2, Oruni, 2023c). Nonetheless, PBO-LLINs still offered a better overall protective advantage compared to conventional LLINs (RR=1.63, 95% CI; 1.29-2.05, P<0.00001) (Gleave *et al.*, 2021). However, all the studies considered in this analysis were from west Africa, which may not be comparable

to the situation in east Africa given the differences in insecticide resistance profiles and intensity (Barrimi *et al.*, 2013; Hancock *et al.*, 2020; Moyes *et al.*, 2020). We observed that the tolerance to PBO is more pronounced in combination with permethrin than deltamethrin as demonstrated in this research as well as previous studies (Mawejje *et al.*, 2013b; Okia *et al.*, 2018), which is possibly due to the concentration of insecticide and PBO used in PermaNet compared to Olyset (WHO, 2009; WHO, 2012).

Our analysis of gene expression showed that the intense pyrethroid resistance observed in Busia is likely driven by expression of multiple metabolic genes, majorly P450s like CYP6P4, CYP6AA1, CYP6P3, CYP6Z3. This is consistent with what has been previously reported in resistant African mosquito vectors (Edi *et al.*, 2014; Ingham *et al.*, 2018; Irving *et al.*, 2012; Mulamba *et al.*, 2014b; Stica *et al.*, 2019b; Wilding *et al.*, 2014). Insecticide resistance in malaria vectors is known to be polygenic, where combinations of several genes are responsible for a resistance phenotype (Ffrench-Constant, 2013). In most cases, the expression of the majority of these key genes conferring resistance are under induced expression while a few genes may be under constitutive expression (Vontas *et al.*, 2005). For example, we noted that on top of the key P450s, the expression of some GSTs and COEs in Busia mosquitoes was induced, except for the CYP6M2 gene that had constitutive expression. This is consistent with what was reported by Djègbè *et al.* (Djègbè *et al.*, 2014), who also showed that even increased exposure time did not increase the expression levels of CYP6M2.

Gene expression data from mosquitoes that survived PBO exposure indicated that there was induced overexpression of only GSTs such as of GSTMS3, GSTE5, GSTD1 and down regulation of the major P450s, compared to unexposed mosquitoes. Most of these GSTs are poorly characterized in *An. gambiae* resistance. Contrary to our findings, other studies have shown that exposure to PBO induced (rather than suppress) gene expression of P450s; for instance, in mice liver (Watanabe *et al.*, 1998) and *Drosophila melanogaster* males (Willoughby *et al.*, 2007). Besides the fact that there is a huge difference in complexity of systems between rodents and arthropods (pointing to different signaling pathways), none of the P450s reported in both studies were within the groups of sub-families implicated in insecticide resistance in malaria vectors. Hence, although PBO may increase gene expression of some P450s, there is currently no evidence that it induces the over-expression of any of the major genes involved in insecticide resistance as available data rather suggests the opposite (Churcher *et al.*, 2016; Fadel *et al.*, 2019; Mawejje *et al.*, 2013b; Snoeck *et al.*, 2017). It is possible that the major P450s involved in insecticide resistance that have been selected over time, also happen to have an antagonistic relationship with PBO, although the exact reason why some P450s are downregulated while others are up-regulated remains to be investigated. In insects, PBO is largely known to combine with P450s and other oxidases forming a metabolite-inhibitory complex with enzyme, which effectively increases the potency of insecticides by blocking the metabolic activity of P450s or their isoenzymes that can detoxify the insecticide (Farnham, 1999; Snoeck *et al.*, 2017). Therefore, mosquitoes that have alternative mechanisms or pathways like GSTs that can detoxify the insecticide but also evade the synergistic effects of PBO, could escape insecticide lethality in presence of PBO. Glutathione-s-transferases have been widely reported to be key enzymes strongly associated with insecticide resistance in African malaria vectors (Hemingway *et al.*, 2004; Ingham *et al.*, 2018; Ranson *et al.*, 2000; Riveron *et al.*, 2014; Wilding *et al.*, 2015) and now possibly tolerance/resistance to PBO-pyrethroid combination. Our findings showed that possible resistance to PBO-pyrethroid combination may be mediated by significantly overexpressing closely related GSTs that also happen to interact with other major GSTs like GSTe2. These results therefore suggest that GSTs could be key genes in tolerance/resistance to PBO nets which might impact the efficacy of PBO nets in the future, as selection pressure mounts. Menze *et al* already showed that in *An. funestus*, a GST-mediated mechanism impacted PBO nets in Cameroon leading to loss of bed net efficacy (Menze *et al.*, 2020). In our study, we analysed only 15 candidate genes by qPCR and therefore might have missed out on other key GSTs. We would recommend using RNAseq experiments to study PBO-pyrethroid exposed survivors in order to identify a full array of genes that are not affected by the synergistic effects of PBO and could also help identify possible novel markers which would be key in tracking PBO tolerance/resistance in real time. As a possible vector control strategy, incorporating diethyl maleate (DEM), an inhibitor of GSTs, (Snoeck *et al.*, 2017) into mosaic LLINs could be an explorable option.

Conclusion

We report that resistance to pyrethroids and DDT remains very high in eastern Uganda and is metabolically driven by multiple gene families. The use of LLINs without synergists therefore may not be very effective; PBO-LLINs, especially PermaNet 3.0, should possibly be the main area of focus for Uganda. However, finding mosquitoes that survive insecticides even in presence of PBO is worrying and could impact the efficiency of PBO-based nets in the future if or when resistance to PBO intensifies due to selection pressure. This study has demonstrated that glutathione-s-transferases could be majorly responsible for PBO-pyrethroid tolerance but a wider and more robust study is required to complement our findings. Meanwhile, a possibility of escalating tolerance/resistance to PBO nets should not be under looked; the mechanisms at play need to be studied in-depth and possible genetic markers for tracking its spread identified.

Data availability

Underlying data

figshare: qPCR Ct-values for the different groups of mosquitoes exposed and unexposed to insecticides. https://doi.org/10.6084/m9.figshare.22638916.v1 (Oruni, 2023a).

This project contains qPCR Ct-values extracted from AriaMx machine (Agilent© technologies) as a text report in Microsoft Excel® format. It includes Ct-values used to calculate the

relative fold change for mosquito groups exposed and not exposed to insecticides.

figshare: qPCR AriaMx files for the different groups of mosquitoes exposed and unexposed to insecticides. https://doi.org/10.6084/m9.figshare.22638964.v1 (Oruni, 2023b).

This project contains the AriaMx machine (Agilent© technologies) qPCR file which was used to extract the Ct-values (Oruni, 2023a) into text report in Microsoft Excel® format. It can only be opened using the AriaMx software.

Extended data

figshare: 2022p TBD (Oruni *et al.*) v5_supplementary Figures.docx https://doi.org/10.6084/m9.figshare.22448395.v1 (Oruni, 2023c).

This project contains three figures: Figure S1- Transcription profiles of the most significantly overexpressed major genes studied in the different exposure groups; Figure S2- Time series analysis of An. gambiae mortality rates reveals decreasing ability of PBO nets to kill highly resistant mosquitoes; and Figure S3- STRING protein network analysis reveals interlinkage between the overexpressed genes in mosquitoes exposed to PBO.

figshare: 2022p TBD (Oruni *et al.*) v5_supplementary table1.docx. https://doi.org/10.6084/m9.figshare.22448398.v1 (Oruni, 2023d).

This project contains a Table S1 showing the re-designed primers for genes used in the RT-qPCR for *Anopheles gambiae s.s.*

Acknowledgements

We would also like to appreciate all the staff and administration at IDRC especially from Busia-Masafu hospital, Tororo district hospital and Nagongera hospital for their great assistance in facilitating this project during field collection of mosquitoes. In particular Patrick Obbo and Joseph Okou for assistance in collection of mosquitoes and Kawesa Bumali for his assistance in conducting cone assays. We are extremely grateful to households that allowed us to collect mosquitoes at very early hours of the morning. We would also like to acknowledge and thank the members of Makerere University-CoVAB-Molecular Biology Laboratory and the Liverpool School of Tropical Medicine for logistical and other support provided as we carried out field and laboratory experiments.

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 28 May 2024

https://doi.org/10.21956/wellcomeopenres.23659.r81002

✔ **Salum Azizi**
Kilimanjaro Christian Medical University College, Moshi, Tanzania

I confirm that all the queries that I raised in the review have now been sufficiently addressed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Medical Entomologist, evaluation of new mosquito control products (insecticide-based)

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

Reviewer Report 16 May 2024

https://doi.org/10.21956/wellcomeopenres.23659.r81001

✔ **Ahmed Idowu Omotayo**
Lagos State University of Science and Technology, Ikorodu, Lagos, Nigeria

The concerns I raised with the first version have been adequately addressed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vector Biology

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

Version 1

Reviewer Report 26 February 2024

https://doi.org/10.21956/wellcomeopenres.21493.r72579

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Ahmed Idowu Omotayo
Lagos State University of Science and Technology, Ikorodu, Lagos, Nigeria

The study set out to contribute to our knowledge of insecticide resistance mechanisms in wild populations of malaria vectors especially in Uganda. It also seeks to assess the efficacy and appropriateness of PBO-LLINs for the control of malaria vectors.

They design is great and well planned and the whole study is excellent, but I have few comments and questions.

MAJOR COMMENT
1.) The natural level resistance profile of the BusiaUG, perhaps at G1 is absent except for deltamethrin, any reason for that? Having the resistance profile for all the insecticides from G1 would have been good for comparison and to understand the baseline profile of the colony. I think the issue might be the population from G1 would not be enough to run that, if yes, adding a sentence about that will be good but if No, kindly explain.

MINOR COMMENT
Introduction
1.) The references about association of cytochrome P450 enzymes to pyrethroid resistance has references 10 years upwards. I am of the opinion that there are more recent studies also on this and that would be better to reference. If possible, give recent publications 2019 to 2024 and the references more than 10 years can be deleted. Authors such as Etang, Edi, Omotayo Mohammed and others have more recent publications on this.

2.) "There are reports of mosquitoes surviving PBO-pyrethroid exposure from a number of countries in Africa including Uganda (Gleave *et al.*, 2021)".

I think there should be a comma after this sentence.

Methods
1.) Hope the "arm feeding" method of female adult mosquitoes was properly approved with the

ethical approval?

2.) Under the "WHO bioassay (resistance phenotyping)", the G1 exposed to discriminating dose of deltamethrin, was it the standard WHO insecticide impregnated papers that was used as used for the G9-G11 exposure? If yes, let it be clear that the standard WHO insecticide impregnated papers were used and if not, please describe how the exposure to discriminating dose was done. I am guessing the exposure papers if not standard WHO papers was done be the investigators.

3.) Under the "WHO bioassay (resistance phenotyping)", the 5-10 pools of alive mosquitoes for each insecticide type for gene expression analysis, whats the number of mosquitoes in each pool? Can we add it. If you mean 5-10 mosquitoes were polled together, can we make the sentence clearer?

I think the conclusion is very appropriate and the recommendation is good.

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

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

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

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

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

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vector Biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 04 Apr 2024

Ambrose Oruni

1. The study set out to contribute to our knowledge of insecticide resistance mechanisms in wild populations of malaria vectors especially in Uganda. It also seeks to assess the efficacy

and appropriateness of PBO-LLINs for the control of malaria vectors. They design is great and well planned and the whole study is excellent, but I have few comments and questions. **Response;** We appreciate the reviewer for the kind complements. 2. MAJOR COMMENT 1.) The natural level resistance profile of the BusiaUG, perhaps at G1 is absent except for deltamethrin, any reason for that? Having the resistance profile for all the insecticides from G1 would have been good for comparison and to understand the baseline profile of the colony. I think the issue might be the population from G1 would not be enough to run that, if yes, adding a sentence about that will be good but if No, kindly explain. **Response;** we only captured this at F1 using pyrethroid and PBO nets as indicated in the edited text. We could only access WHO cone assays in the field for use to ascertain the resistance profile of the population. Indeed, we would have liked to conduct a full insecticide resistance profiling but we didn't do this because our major aim was to maximize the G1 numbers to increase the chances of successfully establishing a colony. We therefore used only deltamethrin to give us an idea of how resistant G1 population is. I have added a sentence as suggested to explain this. 3. MINOR COMMENT Introduction 1.) The references about association of cytochrome P450 enzymes to pyrethroid resistance has references 10 years upwards. I am of the opinion that there are more recent studies also on this and that would be better to reference. If possible, give recent publications 2019 to 2024 and the references more than 10 years can be deleted. Authors such as Etang, Edi, Omotayo Mohammed and others have more recent publications on this. **Response;** I think this shouldn't be a problem. Some of the references mentioned that are above 10 years were articles giving a detailed discussion of P450s, other were discovery of P450s in a different Anopheles species and most of the recent articles still refer to some of these earlier papers. We also wanted to capture the timeline in relation to contribution of P450s to insecticide resistance given that temporal changes have been evident. 2.) "There are reports of mosquitoes surviving PBO-pyrethroid exposure from a number of countries in Africa including Uganda (Gleave et al., 2021)". I think there should be a comma after this sentence. **Response;** we appreciate this observation; this has been corrected. 4. Methods 1.) Hope the "arm feeding" method of female adult mosquitoes was properly approved with the ethical approval? **Response;** Yes, we got all ethical approvals to conduct this study. 2.) Under the "WHO bioassay (resistance phenotyping)", the G1 exposed to discriminating dose of deltamethrin, was it the standard WHO insecticide impregnated papers that was used as used for the G9-G11 exposure? If yes, let it be clear that the standard WHO insecticide impregnated papers were used and if not, please describe how the exposure to discriminating dose was done. I am guessing the exposure papers if not standard WHO papers was done be the investigators. **Response;** we appreciate this comment and to clarify further, Standard WHO papers were used for 1x, 5x, and 10 diagnostic doses since they are available on market. However, we prepared the papers for 2x, 3x diagnostic doses using WHO protocol. I have added a statement to reflect this. 3.) Under the "WHO bioassay (resistance phenotyping)", the 5-10 pools of alive mosquitoes for each insecticide type for gene expression analysis, whats the number of mosquitoes in each pool? Can we add it. If you mean 5-10 mosquitoes were polled together, can we make the sentence clearer? **Response;** it is pools of 5-10 mosquitoes. To explain this, each pool is a biological replicate as indicated in the texts. I have clarified this. 5. I think the conclusion is very appropriate and the recommendation is good. **Response;** we appreciate the reviewer for the kind words.

Competing Interests: There are no conflicting interests.

Reviewer Report 01 February 2024

https://doi.org/10.21956/wellcomeopenres.21493.r72587

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Salum Azizi
Kilimanjaro Christian Medical University College, Moshi, Tanzania

1. The observed decrease in egg laying from G1 to G6 was responded by increasing frequency of blood feeding, what was the response? did it restored the egg laying as in G1?
2. Field mosquito collection explained here is for the 384 mosquitoes used for the establishment of the colony (BusiaUG), but information on where and how wild mosquitoes that was compared with BusiaUG in the phenotypic resistance profiling is not explained
3. Generation 12 of BusiaUG was selected using 0.05% Deltamethrin, what was it for?
4. Figure 1 is not clear in the following aspects: Wild, G1 and G9 were tested using different bioassays (cone bioassays using bed nets tested against Busia wild, deltamethrin resistance intensity tube bioassays tested against G1, and a range of insecticides at diagnostic concentration with or without synergist at G9) making it difficult to compare the results between wild and colony mosquitoes. Wild mosquitoes might be close to G1, however G9 is a distant generation from a wild population (it might already been affected with laboratory conditions in the insectary, in terms of fitness, you have observed a decrease of egg laying for example from G1 to G6), having a wild colony as a comparator in panel B and C is therefore important. Is panel A results obtained at the same time as panel B or C?
5. Information on mosquito sampling and preservation for transcription studies is not provided. It is only mentioned that exposed and unexposed mosquitoes were compared to susceptible mosquitoes, but it is not clear how many were taken and whether survivals were separated from the ones which died among the exposed.
6. Some text in Fig 3 are not readable (improve size/readability)
7. The title of your manuscript is centred on "a new resistant An. gambiae colony from Uganda", but the discussion is mainly focused on wild mosquitoes from Busia. Even the introduction lacks justification why in the first place the "new resistant colony" establishment was important.

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

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

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

Partly

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

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

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Medical Entomologist, evaluation of new mosquito control products (insecticide-based)

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 04 Apr 2024

Ambrose Oruni

1. The observed decrease in egg laying from G1 to G6 was responded by increasing frequency of blood feeding, what was the response? did it restored the egg laying as in G1? **Response;** It didn't restore the egg-laying, it made sure that the colony didn't collapse by increasing the number of blood fed mosquitoes hence increasing the chances of obtaining eggs and more adults in the next generation. 2. Field mosquito collection explained here is for the 384 mosquitoes used for the establishment of the colony (BusiaUG), but information on where and how wild mosquitoes that was compared with BusiaUG in the phenotypic resistance profiling is not explained **Response;** The wild mosquitoes were collected from the same area using the same technique during a baseline survey to determine if the area was viable for collecting mosquitoes for colony establishment especially since IRS was done in the neighboring district. After this collection, we also took advantage to assess the baseline resistance profile. Unfortunately, we didn't take note of how many mosquitoes were collected during the baseline. I have made a notice of this. 3. Generation 12 of BusiaUG was selected using 0.05% Deltamethrin, what was it for? **Response;** This was to maintain resistance within the colony into the subsequent generations. 4. Figure 1 is not clear in the following aspects: Wild, G1 and G9 were tested using different bioassays (cone bioassays using bed nets tested against Busia wild, deltamethrin resistance intensity tube bioassays tested against G1, and a range of insecticides at diagnostic concentration with or without synergist at G9) making it difficult to compare the results between wild and colony mosquitoes. Wild mosquitoes might be close to G1, however G9 is a distant generation from a wild population (it might already been affected with laboratory conditions in the insectary, in terms of fitness, you have observed a decrease of egg laying for example from G1 to G6), having a wild colony as a comparator in panel B and C is therefore important. Is panel A

results obtained at the same time as panel B or C? **Response;** I have made some clarification of this. We collected wild mosquitoes at baseline, reared F1s in Uganda and then used WHO cone assays (because that's what was available) to measure the resistance of the wild population. Indeed, at G1, we performed bioassays using deltamethrin response curve only because we didn't want to use too many mosquitoes for colony establishment. The assumption was that there was no significant difference in the resistance profile from wild and G1. We then performed an array of insecticide phenotyping at G9 when we had a colony and a surplus of adults to use. We checked if; the colony hadn't lost resistance (which they didn't) and profile to other insecticides. We couldn't compare with wild because colony establishment was done in Liverpool hence, we couldn't collect more wild mosquitoes. Panel A results were obtained at baseline and panel B was obtained at G1 (about 2 weeks after collection) and panel C were obtained after colony establishment (about 6 months after collection). This has been explained in the figure legend. 5. Information on mosquito sampling and preservation for transcription studies is not provided. It is only mentioned that exposed and unexposed mosquitoes were compared to susceptible mosquitoes, but it is not clear how many were taken and whether survivals were separated from the ones which died among the exposed. **Response;** We mention this under RT-qPCR sub heading in the first paragraph explaining the RNA extraction. We used biological replicates (pools) containing 5-10 mosquitoes. I have clarified this in the text that we only used mosquitoes that survived the exposure i.e. alive. 6. Some text in Fig 3 are not readable (improve size/readability) **Response;** I have submitted a clearer new figure. 7. The title of your manuscript is centred on "a new resistant An. gambiae colony from Uganda", but the discussion is mainly focused on wild mosquitoes from Busia. Even the introduction lacks justification why in the first place the "new resistant colony" establishment was important. **Response1;** The new colony was established from the same wild population with characteristics and resistance profile that was very similar to the wild/field population given the similarity in pyrethroid resistance level at G9-G11. Hence, the colony was used as foundation to understand the resistance patterns in the *An. gambaie* mosquitoes from Busia. **Response 2;** We didn't justify why the colony was established because it was part of a bigger study of the main grant and we simply took advantage to perform additional experiments given the opportunity. The justification was for why we performed the experiments, which I think is clearly stipulated.

Competing Interests: There are no conflicting interests.