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Characterizing pyrethroid resistance and mechanisms in *Anopheles gambiae* (*s.s.*) and *Anopheles arabiensis* from 11 districts in Uganda

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ABSTRACT

Insecticide resistance threatens recent progress on malaria control in Africa. To characterize pyrethroid resistance in Uganda, *Anopheles gambiae* (*s.s.*) and *Anopheles arabiensis* were analyzed from 11 sites with varied vector control strategies. Mosquito larvae were collected between May 2018 and December 2020. Sites were categorized as receiving no indoor-residual spraying ('no IRS', $n = 3$); where IRS was delivered from 2009 to 2014 and in 2017 and then discontinued ('IRS stopped', $n = 4$); and where IRS had been sustained since 2014 ('IRS active', $n = 4$). IRS included bendiocarb, pirimiphos methyl and clothianidin. All sites received long-lasting insecticidal nets (LLINs) in 2017. Adult mosquitoes were exposed to pyrethroids; with or without piperonyl butoxide (PBO). *Anopheles gambiae* (*s.s.*) and *An. arabiensis* were identified using PCR. *Anopheles gambiae* (*s.s.*) were genotyped for *Vgsc-995S/F*, *Cyp6aa1*, *Cyp6p4-I236M*, *ZZB-TE*, *Cyp4j5-L43F* and *Coeae1d*, while *An. arabiensis* were examined for *Vgsc-1014S/F*. Overall, 2753 *An. gambiae* (*s.l.*), including 1105 *An. gambiae* (*s.s.*) and 1648 *An. arabiensis* were evaluated. Species composition varied by site; only nine *An. gambiae* (*s.s.*) were collected from 'IRS active' sites, precluding species-specific comparisons. Overall, mortality following exposure to permethrin and deltamethrin was 18.8% (148/788) in *An. gambiae* (*s.s.*) and 74.6% (912/1222) in *An. arabiensis*. Mortality was significantly lower in *An. gambiae* (*s.s.*) than in *An. arabiensis* in 'no IRS' sites (permethrin: 16.1 *vs* 67.7%, $P < 0.001$; deltamethrin: 24.6 *vs* 83.7%, $P < 0.001$) and in 'IRS stopped' sites (permethrin: 11.3 *vs* 63.6%, $P < 0.001$; deltamethrin: 25.6 *vs* 88.9%, $P < 0.001$). When PBO was added, mortality increased for *An. gambiae* (*s.s.*) and *An. arabiensis*. Most *An. gambiae* (*s.s.*) had the *Vgsc-995S/F* mutation (95% frequency) and the *Cyp6p4-I236M* resistance allele (87%), while the frequency of *Cyp4j5* and *Coeae1d* were lower (52% and 55%, respectively). Resistance to pyrethroids was widespread and higher in *An. gambiae* (*s.s.*). Where IRS was active, *An. arabiensis* dominated. Addition of PBO to pyrethroids increased mortality, supporting deployment of PBO LLINs. Further surveillance of insecticide resistance and assessment of associations between genotypic markers and phenotypic outcomes are needed to better understand mechanisms of pyrethroid resistance and to guide vector control.

1. Introduction

Remarkable progress in malaria control has been achieved over the past two decades following the scale-up of vector control interventions including long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (Bhatt et al., 2015; Cibulskis et al., 2016; WHO, 2021). Nearly 70% of clinical malaria cases averted between 2000 and 2015 were attributed to use of LLINs (Bhatt et al., 2015). LLINs have been shown to reduce parasite prevalence, malaria morbidity, and malaria mortality in children (Kleinschmidt et al., 2018; Pryce et al., 2018); more recently, use of LLINs in early childhood has been associated with better survival outcomes through adulthood (Fink et al., 2022). In Uganda,

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LLINs serve as the backbone of malaria control, and mass campaigns are conducted every 3–4 years to distribute LLINs nationwide, supplemented by a targeted IRS program (Uganda National Malaria Control Division, 2019). IRS conducted in high-transmission areas has also been very effective (Katureebe et al., 2016; Nankabirwa et al., 2020; Namuganga et al., 2021). Various non-pyrethroid insecticides have been deployed, including bendiocarb (a carbamate), pirimiphos-methyl (an organophosphate) and clothianidin (a neonicotinoid), all with differing modes of action found to be suitable alternatives to pyrethroids (Akogbéto et al., 2010; Agossa et al., 2014; Fongnikin et al., 2020). However, the substantial benefits of LLINs and IRS are threatened by widespread insecticide resistance in Uganda (Mawejje et al., 2013; Mulamba et al., 2014; Okia et al., 2018; Tchouakui et al., 2021), and elsewhere (Ochomo et al., 2013, 2014; Yipmo et al., 2022).

The long-term application of insecticides for public health (WHO, 2021) and control of agricultural pests (Nkya et al., 2014) has increased selection pressure on malaria vectors (Lines, 1988; Nauen, 2007; Mathias et al., 2011; Ranson & Lissenden, 2016), driving the development and spread of insecticide resistance (Mathias et al., 2011; Ranson et al., 2011; Ranson & Lissenden, 2016; Hancock et al., 2020; Wat'senga et al., 2020). Conventional LLINs prequalified by the World Health Organization (WHO) rely on pyrethroid insecticides, including permethrin and deltamethrin, which are favored because of low mammalian toxicity (WHO, 1999), excito-repellency (Elliott et al., 1978; WHO, 2011), and relatively low cost compared to alternative insecticides (Hancock et al., 2020). Mosquitoes with relevant resistance mutations are more likely to survive if exposed to insecticides, thus extending their lifespan and the likelihood of transmitting malaria parasites (Verhaeghen et al., 2010; Kabula et al., 2016). Pyrethroid resistance has been shown to compromise vector control (Kigozi et al., 2012; Toé et al., 2014; Hargreaves et al., 2000), although the impact of insecticide resistance on malaria metrics is less conclusive (Kleinschmidt et al., 2018). Widespread resistance to pyrethroids has been reported across sub-Saharan Africa (Hancock et al., 2020; Lissenden et al., 2021), including in Uganda (Verhaeghen et al., 2006, 2010; Ramphul et al., 2009; Mawejje et al., 2013; Okia et al., 2013, 2018; Katureebe et al., 2016). To combat the spread of pyrethroid resistance, newer generation LLINs have been developed, which incorporate additional chemicals into the nets, such as piperonyl butoxide (PBO), a synergist (WHO, 2017; Protopopoff et al., 2018; Staedke et al., 2020; Gleave et al., 2021), pyriproxyfen, an insect growth regulator (Tiono et al., 2018; Ngufor et al., 2020), and chlorfenapyr, a pyrrole insecticide (Mosha et al., 2022). Initial studies of these dual active-ingredient nets are promising (Mosha et al., 2022). Current WHO guidelines on malaria control (WHO, 2022) recommend deployment of PBO-LLINs in areas with pyrethroid resistance and strategic co-deployment of LLINs and non-pyrethroid IRS, as a strategy to limit insecticide resistance (WHO, 2014, 2015, 2022). Further evidence of the impact of combining LLINs with IRS using non-pyrethroid insecticides on malaria burden and the selection for pyrethroid resistance is needed.

Resistance to pyrethroids is primarily mediated by changes in the voltage-gated sodium channel (*Vgsc*) (Ranson & Lissenden, 2016), which serves as the target site for these insecticides, and through metabolic mechanisms (Donnelly et al., 2009). Non-synonymous point mutations in *Vgsc*, commonly referred to as knockdown resistance (*kdr*) (Martinez-Torres et al., 1998), most commonly involve either an *L995S* (Ranson et al., 2000) or *L995F* (Martinez-Torres et al., 1998) mutation (numbering for *An. gambiae* (*s.s.*); the orthologous codon in *An. arabiensis* is *1014*). Both mutations have been described previously in Uganda, with the *L995S* mutation at greater frequency (Verhaeghen et al., 2006; Mawejje et al., 2013; Okia et al., 2013, 2018; Lynd et al., 2019). Metabolic resistance in *An. gambiae* (*s.s.*) is often associated with changes in cytochrome p450 enzymes that potentially increase insecticide detoxification; in Uganda these include *Cyp4j5* (Weetman et al., 2018), *Cyp6p4* and an associated 'Zanzibar-like' transposable element (*ZZB-TE*) (Njoroge et al., 2021), and the *Cyp6aa1/Cyp6aap* duplication (Lucas et al., 2019; Njoroge et al., 2021). A carboxylesterase gene (*Coeae1d*) (Weetman et al., 2018) has also been associated with pyrethroid resistance in Uganda and Kenya. Previous analysis of *An. gambiae* (*s.s.*) mosquitoes collected from Uganda and Kenya (Weetman et al., 2018) showed that marker polymorphisms in *Cyp4j5* and *Coeae1d* were found at relatively high frequency (0.61 and 0.53, respectively) and were associated with pyrethroid resistance. In Uganda and parts of the Democratic Republic of the Congo, the *Cyp6aa1* duplication, *Cyp6p4* point mutation and *ZZB-TE* insertion are found at high frequency as a triple-mutant (Njoroge et al., 2021), with the two p450 genes shown to be capable of metabolizing pyrethroids *in vitro* in *An. gambiae* (*s.s.*) None of these mechanisms are known to be associated with resistance to the insecticides (bendiocarb, pirimiphos-methyl and clothianidin) used for recent IRS in Uganda. To further characterize pyrethroid resistance in Uganda and explore patterns associated with non-pyrethroid IRS, we collected *An. gambiae* (*s.s.*) and *An. arabiensis* from 11 districts around Uganda under conditions of varying malaria control, including sites with and without IRS programmes, and analysed them using both phenotypic and genotypic assays.

2. Materials and methods

2.1. Study site characteristics

This study was conducted in 11 districts across Uganda (Fig. 1). Mubende and Kayunga districts are located in the central region (North Buganda sub-region), characterised by forest-savannah mosaic vegetation (Roberts & Ocaya, 2009); prevalence of malaria parasitemia in children aged 0–59 months, as measured by microscopy, was 9% in the 2019 Malaria Indicator Survey (MIS) (Uganda National Malaria Control Division, 2019). Kole, Otuke, Dokolo and Amolatar districts are located in the Lango sub-region of northern Uganda, which is characterised by short grassland vegetation (Roberts & Ocaya, 2009), and a regional parasite prevalence of 13% in 2019 (Uganda National Malaria Control Division, 2019). Amuru, Lamwo and Agago districts are located in Acholi sub-region, also in northern Uganda, bordering South Sudan, with a parasite prevalence of 12% in 2019. Busia and Tororo districts are located in Bukedi sub-region in eastern Uganda, bordering western Kenya. This area is characterized by moist savannah vegetation (Roberts & Ocaya, 2009), and parasite prevalence of 3% in 2019 (Uganda National Malaria Control Division, 2019). Previous meteorological data demonstrated that districts in the central and eastern regions experience bimodal rainfall with two peaks, one in March-May and the second in September-December (MOH, 2014), whilst the northern region receives less rainfall, with only one rainy season between March and October (MOH, 2014).

Study sites were stratified by vector control status. In all 11 districts, two mass campaigns were conducted to deliver conventional (pyrethroid only) LLINs in 2013–2014 and in 2017 (Fig. 2). 'No IRS' sites (Busia, Mubende and Kayunga) received LLINs only; the Ministry of Health did not implement IRS in these areas. 'IRS stopped' sites (Kole, Amuru, Lamwo and Agago) received LLINs plus annual rounds of IRS from 2009 to 2014, followed by a single round of IRS in 2017. 'IRS active' sites (Otuke, Tororo, Dokolo and Amolatar) received LLINs plus routine IRS from 2014 to 2019 (active at the time of larval sampling). Details of insecticides used are provided in Fig. 2 and have also been described elsewhere (Namuganga et al., 2021). Briefly, both 'IRS stopped' and 'IRS active' districts received IRS with two insecticide compounds, namely bendiocarb followed by pirimiphos methyl. Dokolo received IRS with clothianidin, rather than pirimiphos methyl, in 2019 (illustrated in Fig. 2).

2.2. Mosquito collections and identification

Mosquito larvae were collected between May 2018 and December 2020 (Fig. 2) using the dipping method (Service, 1993) from a range of breeding sites including man-made pits to excavate sand, brick, or

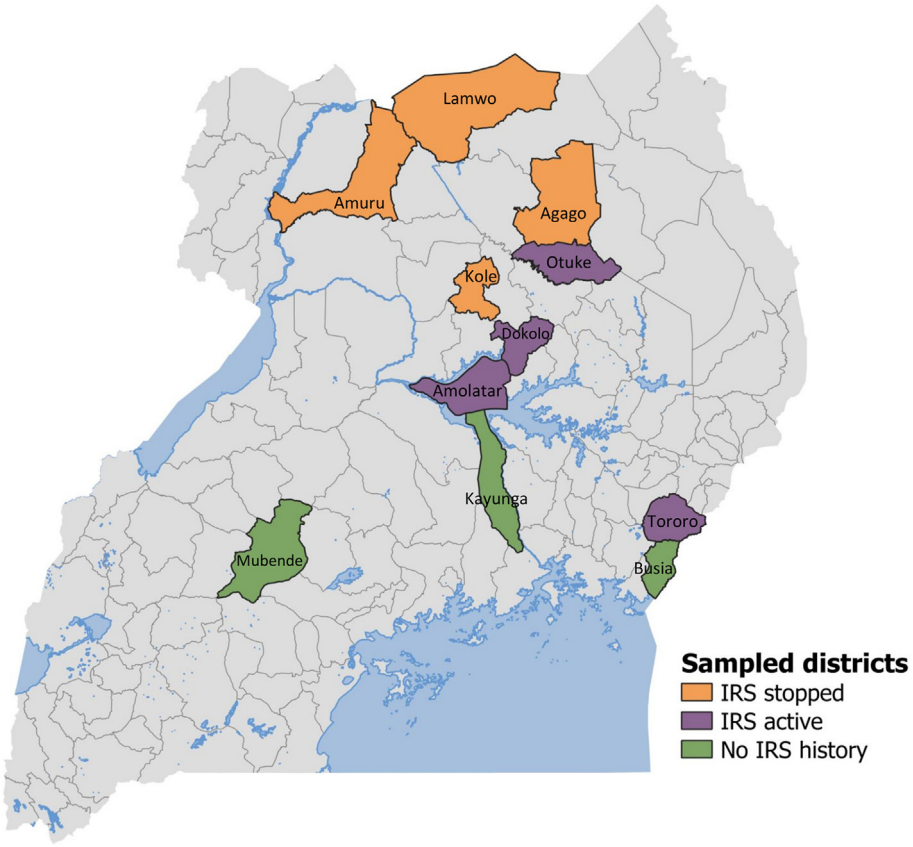

Fig. 1. Map of study sites showing the location of sampled districts, and stratification by vector control measures. *Abbreviations*: IRS, indoor residual spraying; LLINs, long-lasting insecticidal nets. *Key*: green, No IRS (LLINs only); orange, IRS stopped (+ LLINs); purple, IRS active (+ LLINs).

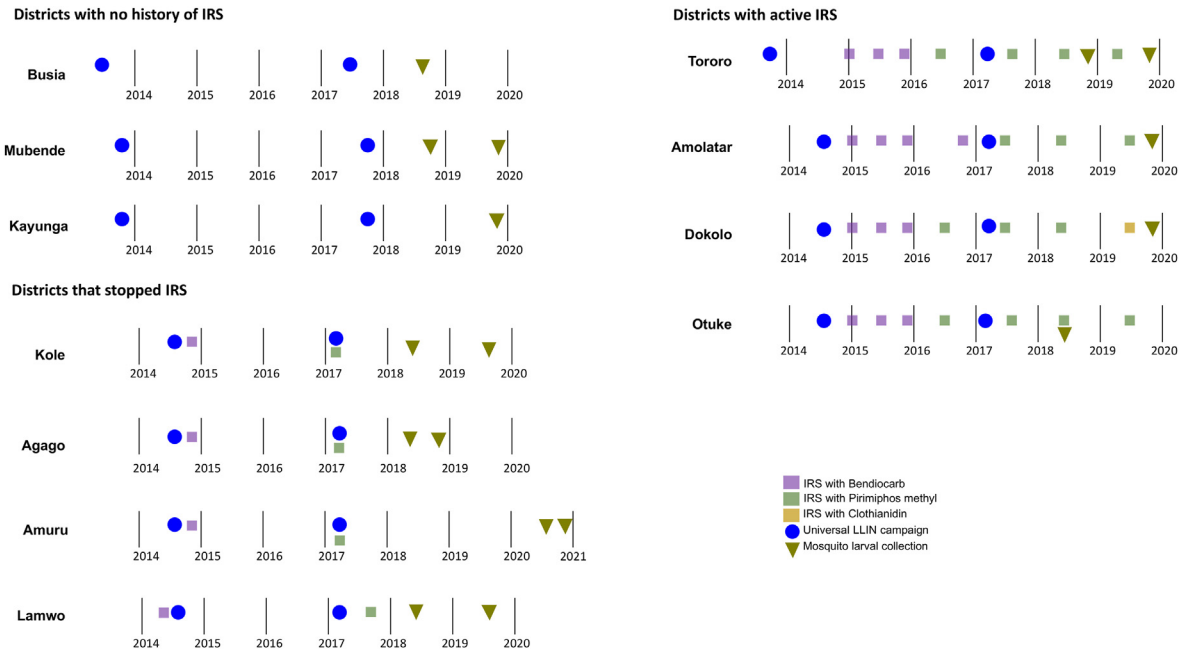

Fig. 2. Timeline of vector control measures and mosquito larval collections in study sites, stratified by IRS status. *Abbreviations*: IRS, indoor residual spraying; LLINs, long-lasting insecticidal nets. *Key*: purple, IRS with bendiocarb; green, IRS with pirimiphos methyl (Actellic); gold, IRS with Sumishield 50W (clothianidin); blue circle, LLINs distributed nationwide by Uganda's Ministry of Health through the 2017–2018 universal coverage campaign; inverted triangles, mosquito larval collection.

murram, cow watering holes, tyre tracks, stagnant roadside pools, rice fields, and harvested gardens. Larvae were transported to the medical entomology insectary at the Central Public Health Laboratories in Kampala and were raised to adults using finely ground Tetramin fish food. Resultant adult mosquitoes were identified morphologically using keys (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987) and classified as members of the *Anopheles gambiae* (*sensu lato*) species complex. Subsequent identification of sibling species was done using standard polymerase chain reaction (PCR) protocols (Scott et al., 1993).

2.3. Insecticide susceptibility tests

Assessment of insecticide susceptibility was performed using standard WHO tube bioassays (WHO, 1998, 2016). Adult non-blood-fed female *An. gambiae* (*s.l.*), aged 3–5 days-old were exposed to permethrin or deltamethrin at WHO diagnostic concentrations of 0.75% and 0.05%, respectively. Four replicates of 20–25 mosquitoes were exposed per insecticide for 1 h under temperatures ranging from 23.3 °C to 26.7 °C and relative humidity between 80% and 95%. Mortality was scored 24-h post-insecticide exposure. Mosquito samples were stored individually and preserved using desiccant silica gel for subsequent molecular analysis. For quality control, each assay was run with a control tube of 20–25 mosquitoes containing (standard pyrethroid control) silicone oil papers. Phenotypic data from larvae collected at different sampling points (Fig. 2) were pooled within each study site to improve test power.

2.4. Synergist bioassays

To further investigate underlying mechanisms of pyrethroid resistance *via* the synergist PBO, which acts primarily to block detoxification by cytochrome P450 monooxygenases, adult female *An. gambiae* (*s.l.*) were exposed to WHO insecticide papers treated with PBO (4%) for 1 h followed by permethrin or deltamethrin exposure for an additional diagnostic period of 1 h. Mortality was scored after 24 h. In control samples, PBO control papers were used prior to pyrethroid control paper exposure. Mosquito samples were stored singly over silica gel for further molecular analysis.

2.5. Molecular analysis

Genomic DNA was extracted from whole mosquitoes using the DNeasy kit (Qiagen, Hilden, Germany) and used as a template for molecular analyses. The *Vgsc* genotype at codon *1014* (*995* using *An. gambiae* (*s.s.*) numbering) (The Anopheles gambiae 1000 Genomes Consortium, 2017) were determined using a locked nucleic allele (LNA) assay, which detects wild type and *kdr* mutants serine or phenylalanine (Lynd et al., 2018). The triple mutation with *Cyp6aa1* duplication, *Cyp6p4-I236M* and *ZZB-TE* (cytochrome p450-linked 'Zanzibar-like' transposable element) was assessed using three independent LNA assays (Njoroge et al., 2021). All assays were run on AriaMx Real-Time PCR machine (Agilent, Santa Clara, USA). TaqMan assays were used to genotype *Cyp4j5* and *Coeae1d* (Weetman et al., 2018). TaqMan assays used a primer/probe mix in addition to 1× sensimix (Bioline) and DNA template (1 μl) in a 10 μl volume reaction with denaturing for 5 min at 95 °C, followed by 40 cycles of denaturing for 15 s at 92 °C and annealing for 1 min at 60 °C. The TaqMan assays were performed on an Agilent MX3005P Real-Time PCR machine.

2.6. Statistical analysis

Statistical analysis using Stata (version 14.2, Stata Corp, College Station, TX, USA) generated measures of association (odds ratios) using mixed effects logistic regression, adjusting for repeated observations from the same study site. Key exposure variables were insecticide exposure, IRS status and species status. The primary outcome was mosquito phenotype, assessing whether changes in the exposure resulted in mortality or survival. To examine associations between genotypic markers of resistance and phenotypic outcomes, a logistic regression model was used. The nonsynonymous point mutation *Cyp6p4* was selected as the marker of reference in the triple mutant haplotype due to the high level of correlation. Data were pooled by site and categorized by IRS status to improve the statistical power of the model. Pyrethroid resistance markers included in the model were *Vgsc-L995S*, *Vgsc-L995F*, *Cyp6p4-I236M*, *Cyp4j5-L43F* and *Coeae1d*.

3. Results

3.1. Species composition

Overall, 2753 *An. gambiae* (*s.l.*) adults were raised from larvae collected in 11 sites were phenotyped for pyrethroid resistance and speciated, including 1105 *An. gambiae* (*s.s.*) and 1648 *An. arabiensis* (Table 1). In the 'no IRS' sites, where vector control was limited to LLINs, the proportion of mosquitoes identified as *An. gambiae* (*s.s.*) ranged between 33.6 and 83.8%, while *An. arabiensis* ranged between 16.2 and 66.4%. In the sites where IRS was stopped 1.8–3.8 years prior to completing larval collections, most mosquitoes were identified as *An. gambiae* (*s.s.*) at 3 sites (76.5–99.4%), but at one site (Agago) 100% of mosquitoes were *An. arabiensis*. In the four IRS-active sites, in which IRS had been sustained for at least 3.5 years prior to larval collection, nearly all mosquitoes were identified as *An. arabiensis* (98–100%); only nine *An. gambiae* (*s.s.*) were collected from sites with active IRS, and these were excluded from subsequent analyses due to the small sample size.

3.2. Phenotypic bioassay results stratified by IRS categories, mosquito species, and insecticides

Anopheles gambiae (*s.s.*) and *An. arabiensis* were exposed to diagnostic concentrations of permethrin and deltamethrin, and mortality was measured (Supplementary Table S1). Overall, mortality of *An. gambiae* (*s.s.*) following exposure to pyrethroids was low, indicating high prevalence of resistance: 12.9% (53/411) for permethrin and 25.2% (95/377) for deltamethrin. Mortality of *An. arabiensis* was higher, indicating greater susceptibility to pyrethroids: 65.5% (402/614) for permethrin and 82.4% (510/619) for deltamethrin. Phenotypic assay results were pooled and compared between IRS category, species, and insecticide (Table 2). When different IRS category sites were compared, no significant difference in mortality was observed after exposure to either permethrin and deltamethrin, for either *An. gambiae* (*s.s.*) or *An. arabiensis*. When mosquito species were compared, mortality after exposure to both permethrin and deltamethrin was significantly lower for *An. gambiae* (*s.s.*) than *An. arabiensis* in both 'no IRS' (16.1 *vs* 67.7%, $P < 0.001$ for permethrin; 24.6 *vs* 83.7%, $P < 0.001$ for deltamethrin) and 'IRS stopped' sites (11.3 *vs* 63.6%, $P < 0.001$ for permethrin; 25.6 *vs* 88.9%, $P < 0.001$ for deltamethrin). In the 'IRS active' sites, the limited number of *An. gambiae* (*s.s.*) precluded species-specific comparisons. When the two pyrethroids were compared, *An. gambiae* (*s.s.*) mortality was significantly lower following exposure to permethrin than to deltamethrin in 'IRS stopped' sites (11.3 *vs* 25.6%, $P = 0.001$), but not in 'no IRS' sites (16.1 *vs* 24.6%, $P = 0.10$). For *An. arabiensis*, mortality was significantly lower following exposure to permethrin than to deltamethrin in 'no IRS' sites (67.7 *vs* 83.7%, $P = 0.002$), 'IRS stopped' sites (63.6 *vs* 88.9%, $P < 0.001$), and 'IRS active' sites (65.4 *vs* 79.2%, $P < 0.001$).

3.3. Synergist bioassays with piperonyl butoxide

Overall, when *An. gambiae* (*s.s.*) were exposed to the synergist PBO, mortality to both pyrethroids increased (Supplementary Table S1); for permethrin from 12.9% (53/411) to 56.5% (96/170), and for deltamethrin from 25.2% (95/377) to 68.7% (101/147). In *An. arabiensis*, mortality following PBO exposure also increased, from 65.5% (402/614)

Table 1
Mosquitoes tested using phenotypic assays stratified by species, insecticide exposure, study site and vector control measures.

Species	Insecticide exposure	No IRS			IRS stopped				IRS active			
		Busia	Mubende	Kayunga	Kole	Amuru	Lamwo	Agago	Otuke	Tororo	Dokolo	Amolatar
An. gambiae (*s.s.*)	Total	58	171	135	241	169	322	0	0	0	6	3
	Permethrin	32	66	26	113	89	82	0	0	0	2	1
	Permethrin + PBO	0	16	39	0	39	75	0	0	0	1	0
	Deltamethrin	26	74	22	128	41	81	0	0	0	3	2
	Deltamethrin + PBO	0	15	48	0	0	84	0	0	0	0	0
An. arabiensis	Total	71	33	267	74	1	81	158	112	365	293	193
	Permethrin	34	15	78	41	0	16	72	57	119	86	96
	Permethrin + PBO	0	1	49	0	1	15	0	0	70	73	0
	Deltamethrin	37	17	93	33	0	16	86	55	111	74	97
	Deltamethrin + PBO	0	0	47	0	0	34	0	0	65	60	0

Table 2
Mosquito mortality after exposure to pyrethroid insecticides using phenotypic assays, stratified by species, IRS category and insecticide.

Comparison between IRS category, stratified by species							
Species	IRS category	Permethrin			Deltamethrin		
		Mortality (%)	Odds ratio (95% CI)	*P*-value	Mortality (%)	Odds ratio (95% CI)	*P*-value
An. gambiae (*s.s.*)	No IRS	20/124 (16.1)	Reference		30/122 (24.6)	Reference	
	IRS stopped	32/284 (11.3)	0.64 (0.15–2.71)	0.55	64/250 (25.6)	0.66 (0.19–2.33)	0.52
An. arabiensis	No IRS	86/127 (67.7)	Reference		123/147 (83.7)	Reference	
	IRS stopped	82/129 (63.6)	0.90 (0.28–2.94)	0.86	120/135 (88.9)	1.37 (0.51–3.64)	0.53
	IRS active	234/358 (65.4)	1.39 (0.47–4.10)	0.55	267/337 (79.2)	0.83 (0.37–1.87)	0.66
Comparison between mosquito species, stratified by IRS category							
IRS category	Species	Permethrin			Deltamethrin		
		Mortality (%)	Odds ratio (95% CI)	*P*-value	Mortality (%)	Odds ratio (95% CI)	*P*-value
No IRS	*An. arabiensis*	86/127 (67.7)	Reference		123/147 (83.7)	Reference	
	An. gambiae (*s.s.*)	20/124 (16.1)	0.10 (0.05–0.19)	< 0.001	30/122 (24.6)	0.06 (0.03–0.12)	< 0.001
IRS stopped	*An. arabiensis*	82/129 (63.6)	Reference		120/135 (88.9)	Reference	
	An. gambiae (*s.s.*)	32/284 (11.3)	0.20 (0.10–0.38)	< 0.001	64/250 (25.6)	0.08 (0.03–0.18)	< 0.001
Comparison between insecticides, stratified by IRS category							
IRS category	Insecticide	*An. gambiae* (*s.s.*)			*An. arabiensis*		
		Mortality (%)	Odds ratio (95% CI)	*P*-value	Mortality (%)	Odds ratio (95% CI)	*P*-value
No IRS	Deltamethrin	30/122 (24.6)	Reference		123/147 (83.7)	Reference	
	Permethrin	20/124 (16.1)	0.59 (0.31–1.11)	0.10	86/127 (67.7)	0.40 (0.22–0.72)	0.002
IRS stopped	Deltamethrin	64/250 (25.6)	Reference		120/135 (88.9)	Reference	
	Permethrin	32/284 (11.3)	0.44 (0.27–0.71)	0.001	82/129 (63.6)	0.21 (0.11–0.41)	< 0.001
IRS active	Deltamethrin	Insufficient *An. gambiae* (*s.s.*) collected			267/337 (79.2)	Reference	
	Permethrin				234/358 (65.4)	0.48 (0.34–0.68)	< 0.001

to 93.3% (195/209) for permethrin, and from 82.4% (510/619) to 89.8% (185/206) for deltamethrin. In the 'no IRS' sites, mortality of *An. gambiae* (*s.s.*) was significantly higher when PBO was added compared to that with the pyrethroid alone (permethrin: 54.5 *vs* 16.1%, $P < 0.001$; deltamethrin: 55.6 *vs* 24.6%, $P < 0.001$), indicating at least partial restoration of susceptibility to both permethrin and deltamethrin by PBO (Table 3). Similar results were observed in the 'IRS stopped' sites (permethrin: 57.0 *vs* 11.3%, $P < 0.001$; deltamethrin: 78.6 *vs* 25.6%, $P < 0.001$). When *An. arabiensis* from the 'no IRS' sites were exposed to PBO, mortality increased slightly, but not significantly, with permethrin (82.0 *vs* 67.7%, $P = 0.36$). Unexpectedly, mortality following exposure to PBO and deltamethrin was significantly lower compared to that with deltamethrin alone (66.0 *vs* 83.7%, $P = 0.01$). When *An. arabiensis* from the 'IRS stopped' sites were exposed to PBO, mortality increased to 100% for

Table 3
Mosquito mortality after exposure to pyrethroid insecticides with and without piperonyl butoxide, by species and IRS category.

IRS category	Insecticide	*An. gambiae* (*s.s.*)			*An. arabiensis*		
		Mortality (%)	Odds ratio (95% CI)	*P*-value	Mortality (%)	Odds ratio (95% CI)	*P*-value
No IRS	Permethrin	20/124 (16.1)	Reference		86/127 (67.7)	Reference	
	Permethrin + PBO	30/55 (54.5)	6.81 (3.08–15.1)	< 0.001	41/50 (82.0)	1.52 (0.62–3.70)	0.36
	Deltamethrin	30/122 (24.6)	Reference		123/147 (83.7)	Reference	
	Deltamethrin + PBO	35/63 (55.6)	3.83 (2.01–7.31)	< 0.001	31/47 (66.0)	0.38 (0.18–0.80)	0.01
IRS stopped	Permethrin	32/284 (11.3)	Reference		82/129 (63.6)	Reference	
	Permethrin + PBO	65/114 (57.0)	15.0 (7.23–31.2)	< 0.001	16/16 (100)	Omitted because of collinearity	
	Deltamethrin	64/250 (25.6)	Reference		120/135 (88.9)	Reference	
	Deltamethrin + PBO	66/84 (78.6)	18.1 (8.36–39.3)	< 0.001	34/34 (100)	Omitted because of collinearity	
IRS Active	Permethrin	Insufficient *An. gambiae* (*s.s.*) collected			234/358 (65.4)	Reference	
	Permethrin + PBO				138/143 (96.5)	16.1 (6.31–41.2)	< 0.001
	Deltamethrin				267/337 (79.2)	Reference	
	Deltamethrin + PBO				120/125 (96.0)	7.37 (2.82–19.3)	< 0.001

both permethrin and deltamethrin, but statistical significance could not be determined because all *An. arabiensis* died and comparisons could not be made. In the 'IRS active' sites, mortality of *An. arabiensis* increased significantly when PBO was added to both permethrin (96.5 *vs* 65.4%, $P < 0.001$) and deltamethrin (96.0 *vs* 79.2%, $P < 0.001$).

3.4. Molecular markers of insecticide resistance in An. gambiae (s.s.)

A subset of *An. gambiae* (*s.s.*) (Supplementary Table S2) were genotyped for molecular markers associated with pyrethroid resistance, including the *kdr* target site mutations *Vgsc-L995S* and *Vgsc-L995F*, and *Cyp6aa1*, *Cyp6p4*, *ZZB-TE*, *Cyp4j5* and *Coeae1d*, associated with metabolic resistance. The frequency of the *Vgsc-L995S* resistance allele was high in the 'no IRS' sites, ranging from 83% in Kayunga to 96% in Busia, but was low in the 'IRS stopped' sites, ranging from 62% in Lamwo to 74% in Kole (Fig. 3, Supplementary Table S3). The frequency of the *Vgsc-L995F* resistance allele was low in *An. gambiae* (*s.s.*) but was highest in the northern 'IRS stopped' sites, ranging from 15% in Kole to 37% in Lamwo (Fig. 3, Supplementary Table S3; a summary of *Vgsc* genotypes in *An. gambiae* (*s.s.*) is shown in Supplementary Table S7). Comparison of resistance allele frequencies showed significantly higher *Vgsc-L995F* frequency in the 'IRS stopped' compared to 'no IRS' sites (28.40 *vs* 3.43, Fisher's exact test, $P = 0.02$). There was no significant difference in *Vgsc-L995S* resistance allele frequencies between the 'IRS stopped' and 'no IRS' sites (Supplementary Table S4). A high level of agreement was found between the metabolic resistance markers *Cyp6aa1*, *Cyp6p4* and *ZZB-TE* (Spearman's rank correlation = 0.72 for *Cyp6p4* and 0.74 for *ZZB-TE* relative to *Cyp6aa1*). Thus, analyses were restricted to *Cyp6p4*. The frequency of the *Cyp6p4-I236M* resistance allele was very high in *An. gambiae* (*s.s.*) from all sites regardless of IRS status, ranging from 80% in Kayunga to 93% in Mubende, while the frequency of *Cyp4j5* and *Coeae1d* ranged from 42% in Amuru to 65% in Kole and from 44% in Mubende to 62% in Amuru, respectively (Fig. 3, Supplementary Table S3).

3.5. Molecular markers of insecticide resistance in An. arabiensis

For *An. arabiensis*, only target-site resistance mutations (*Vgsc-1014S* and *Vgsc-1014F*) were genotyped (Supplementary Tables S5 and S6). *Anopheles arabiensis* were predominantly wild type (*Vgsc-1014L)* for *kdr* (Fig. 4); *Vgsc-1014S* was found only in Kayunga (3%) and in Kole (11%), while *Vgsc-1014F* was found in Agago (1%), Lamwo (2%) and Kole (9%). *Vgsc-1014S* was not detected in *An. arabiensis* from the 'IRS active' sites. However, *Vgsc-1014F* was found in a single *An. arabiensis* mosquito in Tororo and in one other *An. arabiensis* mosquito from Amolatar (Supplementary Table S5). A summary of *Vgsc* genotypes in *An. arabiensis* is provided in Supplementary Table S8).

3.6. Association between genotypic resistance markers and phenotypic assays in An. gambiae (s.s.)

Analysis of the associations between genotypic resistance markers and phenotypic results in *An. gambiae* (*s.s.*) from 'no IRS' sites (Table 4) revealed significant associations between the target site mutations, *Vgsc-L995 S/F*, and survival when exposed to deltamethrin (odds ratio, OR: 3.44; 95% CI: 1.02–11.57; $P = 0.046$) and between *Cyp4j5* and survival when exposed to deltamethrin + PBO (OR: 2.27; 95% CI: 1.08–4.80; $P = 0.031$). In 'IRS stopped' sites (Table 5), significant associations were found between *Cyp6p4* and survival when exposed to permethrin + PBO (OR: 3.19; 95% CI: 1.16–8.80; $P = 0.025$) and when exposed to deltamethrin (OR: 2.27; 95% CI: 1.02–5.05, $P = 0.045$). All other measures of association were found to be non-significant in the 'no IRS' sites (Table 4) and 'IRS stopped' sites (Table 5).

4. Discussion

Resistance to pyrethroid insecticides threatens the effectiveness of malaria vector control. To further characterize pyrethroid resistance in Uganda, we collected *An. gambiae* (*s.l.*) from 11 districts implementing different IRS-based vector control strategies. We found high levels of pyrethroid resistance, particularly in *An. gambiae* (*s.s.*), but in settings where IRS was active, *An. arabiensis* dominated and almost no *An. gambiae* were identified. Combining PBO with a pyrethroid increased mortality for *An. gambiae* (*s.s.*), as well as *An. arabiensis* in some settings, indicating partial restoration of pyrethroid susceptibility and supporting the use of PBO LLINs in Uganda. The underlying genotypes only partially explained the resistance phenotype in *An. gambiae* (*s.s.*), while *An.*

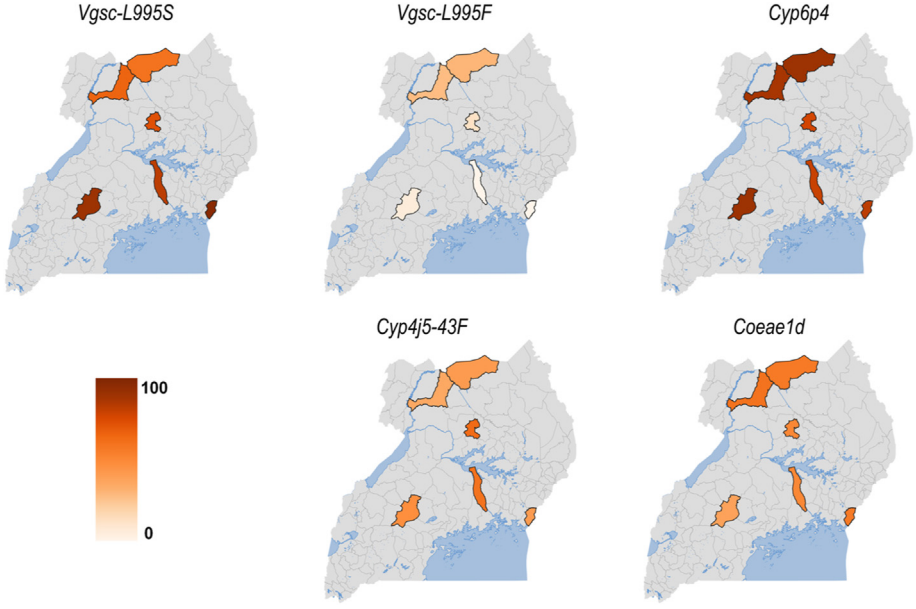

Fig. 3. Heatmaps showing the frequencies of target site mutations *Vgsc-995S* and *Vgsc-995F*, the triple mutant (represented by *Cyp6P4*), a cytochrome p450 *Cyp4j5-L43F* and carboxylesterase *Coeae1d*, associated with resistance to pyrethroids in *An. gambiae* (*s.s.*). The color scale ranges from white (0%) to dark orange (100%); the darker the shade, the higher the resistant allele frequency.

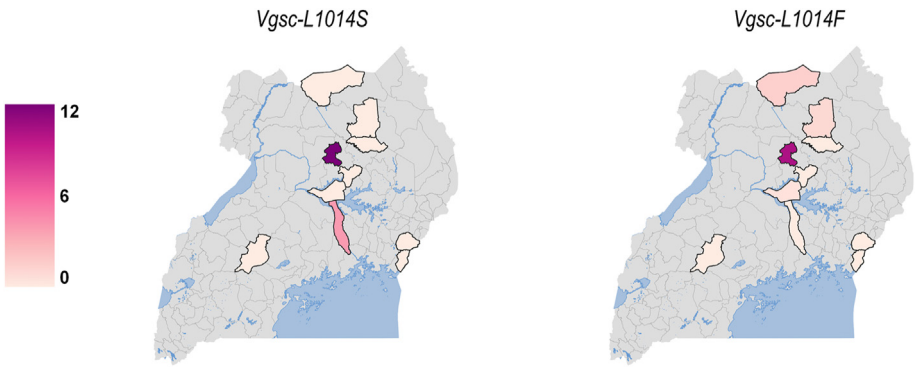

Fig. 4. Heatmaps showing the frequency of target site mutation *Vgsc-L1014S* and *Vgsc-L1014F* in *An. arabiensis*. The color scale ranges from white (0%) to dark purple (12%); the darker the shade, the higher the resistant allele frequency.

Table 4
Associations between resistant alleles and mosquito survival in *An. gambiae* (*s.s.*) mosquitoes following exposure to pyrethroid insecticides with and without piperonyl butoxide in sites with no IRS.

Resistant alleles	Resistant allele frequency *n/N* (%)	Wild type alleles-survived *n/N* (%)	Resistant alleles-survived *n/N* (%)	Odds ratio (95% CI)	*P*-value	Resistant allele frequency *n/N* (%)	Wild type alleles-survived *n/N* (%)	Resistant alleles-survived *n/N* (%)	Odds ratio (95% CI)	*P*-value
	Permethrin					Permethrin + PBO				
Vgsc-L995 S/F	214/218 (98.2)	4/4 (100)	180/214 (84.1)	0.62 (0.17–2.26)	0.46	94/106 (88.7)	2/12 (16.7)	44/94 (46.8)	1.17 (0.23–5.94)	0.85
Cyp6P4 (triple mutant)	204/226 (90.3)	19/22 (86.4)	173/204 (84.8)	0.93 (0.23–3.67)	0.91	87/106 (82.1)	6/19 (31.6)	40/87 (46.0)	1.22 (0.27–5.58)	0.80
Cyp4j5	113/216 (52.3)	83/103 (80.6)	99/113 (87.6)	1.85 (0.80–4.29)	0.15	60/106 (56.6)	22/46 (47.8)	24/60 (40.0)	0.84 (0.33–2.16)	0.72
Coeae1d	107/218 (49.1)	90/111 (81.1)	94/107 (87.9)	1.97 (0.82–4.77)	0.13	51/106 (48.1)	25/55 (45.5)	21/51 (41.2)	0.85 (0.32–2.25)	0.74
	Deltamethrin					Deltamethrin + PBO				
Vgsc-L995 S/F	271/278 (97.5)	2/7 (28.6)	206/271 (76.0)	3.44 (1.02–11.6)	0.046	102/125 (81.6)	14/23 (60.9)	42/102 (41.2)	1.41 (0.40–4.97)	0.59
Cyp6P4 (triple mutant)	248/278 (89.2)	20/30 (66.7)	188/248 (75.8)	0.82 (0.31–2.21)	0.70	95/126 (75.4)	15/31 (48.4)	41/95 (43.2)	0.94 (0.32–2.80)	0.91
Cyp4j5	147/278 (52.9)	102/131 (77.9)	106/147 (72.1)	0.82 (0.46–1.47)	0.51	75/134 (56.0)	15/59 (25.4)	41/75 (54.7)	2.27 (1.08–4.80)	0.03
Coeae1d	141/278 (50.7)	103/137 (75.2)	105/141 (74.5)	0.98 (0.50–1.89)	0.94	63/124 (50.8)	30/61 (49.2)	26/63 (41.3)	0.65 (0.27–1.55)	0.33

arabiensis were predominantly wild type for the target site resistance mutation.

In this study, resistance to permethrin and deltamethrin was widespread. Mortality in phenotypic assays was significantly lower in *An. gambiae* (*s.s.*) than *An. arabiensis* in sites without ongoing IRS. Mortality following exposure to permethrin was significantly lower than to deltamethrin for *An. gambiae* (*s.s.*) in sites where IRS had been stopped (but not in 'no IRS' sites), and for *An. arabiensis* in all sites, suggesting greater resistance to permethrin (a type I pyrethroid) than to deltamethrin (type II). Most *An. gambiae* (*s.s.*) had *Vgsc-995* target site mutations, while these mutations were uncommon in *An. arabiensis*. The *Cyp6p4-I236M* resistance allele, a marker of metabolic resistance, was also common in *An. gambiae* (*s.s.*), while *Cyp4j5* and *Coeae1d* were less common, present in just over half of *An. gambiae* (*s.s.*) tested. Some associations between genotypic markers of resistance and phenotypic outcomes were observed in *An. gambiae* (*s.s.*), although results were inconsistent, suggesting mechanisms of pyrethroid resistance are complex and insufficiently explained by currently recognized resistance markers.

The target site resistance mutation *Vgsc-995S* was found at very high frequency in *An. gambiae* (*s.s.*), consistent with prior observations in Uganda and Kenya (Okia et al., 2018; Lynd et al., 2019). The presence of the *Vgsc-995F* mutation, which has been associated with a strong resistance phenotype (Reimer et al., 2008), suggests pyrethroid selection pressure, in the study sites. The *Vgsc-995F* mutation has also been noted to confer greater resistance to type I (permethrin) than type II (deltamethrin) pyrethroids (Reimer et al., 2008), which may partially account for the significantly lower *An. gambiae* (*s.s.*) mortality to permethrin compared to deltamethrin observed in the 'IRS stopped' but not in the 'no IRS' sites. However, the very low frequency of this mutation (*Vgsc-L1014F* alternative) in *An. arabiensis*, suggests that the observed difference in insecticide specific mortality may be driven by other resistance mechanisms. The prevalence of the *Vgsc-995F* mutation seems to be increasing in Uganda, since the first report of this mutation at very low frequency in *An. gambiae* (*s.s.*) approximately 15 years ago (Verhaeghen et al., 2006). We found *kdr* mutations (*Vgsc-995S* and *Vgsc-995F*) within the same sample, particularly in *An. gambiae* (*s.s.*) The presence of both mutations (F/S heterozygotes) within the same mosquito is associated with a strong pyrethroid resistance phenotype, similar to that of F/F homozygotes. In *An. arabiensis*, both *kdr* mutations (*L1014S* and *L1014F*) were at relatively low frequency, with most individuals wild type homozygotes, akin to findings elsewhere in Uganda (Mawejje et al., 2013; Lynd et al., 2019). Nevertheless, *kdr* mutations (*Vgsc-L1014S*) in *An. arabiensis* have been found at frequencies as high as 63% in mosquitoes from Western Kenya (Hemming-Schroeder et al., 2018),

Table 5
Associations between resistant alleles and mosquito survival in *An. gambiae* (*s.s.*) mosquitoes following exposure to pyrethroid insecticides with and without piperonyl butoxide in sites where IRS was stopped.

Resistant alleles	Resistant allele frequency *n/N* (%)	Wild type alleles-survived *n/N* (%)	Resistant alleles-survived *n/N* (%)	Odds ratio (95% CI)	*P*-value	Resistant allele frequency *n/N* (%)	Wild type alleles-survived *n/N* (%)	Resistant alleles-survived *n/N* (%)	Odds ratio (95% CI)	*P*-value
	Permethrin					Permethrin + PBO				
Vgsc-L995 S/F	424/432 (98.2)	6/8 (75)	370/424 (87.2)	1.40 (0.85–2.30)	0.18	199/202 (98.5)	0/3 (0)	100/199 (50.3)	1.25 (0.77–2.03)	0.37
Cyp6P4 (triple mutant)	400/440 (90.9)	25/40 (62.5)	351/400 (87.8)	1.87 (0.86–4.09)	0.12	175/198 (88.4)	6/23 (26.1)	92/175 (52.6)	3.19 (1.16–8.80)	0.025
Cyp4j5	214/430 (49.8)	189/216 (87.5)	185/214 (86.4)	0.75 (0.22–2.57)	0.64	95/200 (47.5)	49/105 (46.7)	51/95 (53.7)	1.35 (0.80–2.28)	0.27
Coeae1d	254/432 (58.8)	157/178 (88.2)	219/254 (86.2)	0.78 (0.42–1.48)	0.45	119/202 (58.9)	45/83 (54.2)	55/119 (46.2)	0.70 (0.37–1.32)	0.27
	Deltamethrin					Deltamethrin + PBO				
Vgsc-L995 S/F	270/306 (88.2)	6/36 (16.7)	190/270 (70.4)	1.64 (0.99–2.71)	0.056	72/72 (100)	0/0 (0)	36/72 (50)	1.60 (0.67–3.83)	0.30
Cyp6P4 (triple mutant)	256/306 (83.7)	13/50 (26.0)	183/256 (71.5)	2.27 (1.02–5.05)	0.045	67/70 (95.7)	0/3 (0)	36/67 (53.7)	–	–
Cyp4j5	204/306 (66.7)	71/102 (69.6)	125/204 (61.3)	1.0 (0.52–1.91)	0.99	7/70 (10.0)	30/63 (47.6)	4/7 (57.1)	1.19 (0.21–6.74)	0.85
Coeae1d	164/306 (53.6)	84/142 (59.2)	112/164 (68.3)	1.55 (0.83–2.88)	0.17	42/72 (58.3)	14/30 (46.7)	22/42 (52.4)	2.07 (0.64–6.66)	0.22

neighboring Tororo (IRS active) and Busia (No IRS) districts, and as high as 89.5% in *An. arabiensis* from Dakar, Senegal (Dia et al., 2018).

The recently described mutants *Cyp6aa1*, *Cyp6p4* and *ZZB-TE* (Njoroge et al., 2021) were found to be strongly correlated in *An. gambiae* (*s.s.*), indicating strong, though imperfect linkage disequilibrium and a high frequency of the triple mutant haplotype. The triple-mutant (represented by *Cyp6p4*) suggested strong positive selection in geographically distinct *An. gambiae* (*s.s.*) and was found at a frequency ranging from 80 to 93% in the target sites. This is consistent with observations of *An. gambiae* (*s.s.*) collected in Busia, Uganda and in Kenya (Njoroge et al., 2021). The *Cyp6p4* mutation was associated with resistance to deltamethrin, similar to findings from western Kenya described by Njoroge et al. (2021). However, the association between the triple-mutant and mosquito survival following exposure to permethrin and PBO observed in this study has not previously been described and is unexpected given the expected blocking effects of PBO on P450 enzyme activity (Farnham, 1999). However, Njoroge et al. (2021) found that PBO LLINs were effective against a pyrethroid-resistant colony (from Busia, Uganda) with a triple-mutant frequency of 29.7%. The association between the *Cyp4j5* P450 marker, and mosquito survival following exposure to deltamethrin plus PBO is another novel finding and similarly unexpected, although previous reports have found significant association between *Cyp4j5* and deltamethrin (as well as permethrin) resistance (Weetman et al., 2018) and to our knowledge the marker association's relationship with PBO has not previously been assessed.

Cluster-randomized trials in Uganda (Staedke et al., 2020) and Tanzania (Protopopoff et al., 2018) demonstrated significant declines in mosquito density and parasite prevalence associated with PBO LLINs, supported by the recently revised Cochrane review on PBO LLINs (Gleave et al., 2021). The WHO's Vector Control Advisory Group concluded that PBO LLINs are more effective than pyrethroid-only LLINs in settings of high-level pyrethroid resistance, and the WHO now recommends PBO LLINs for the prevention and control of malaria in areas where malaria vectors demonstrate substantial pyrethroid resistance (WHO, 2022). As PBO LLINs are scaled-up, surveillance of markers of metabolic resistance will be essential.

We observed differences in the distribution of *An. gambiae* (*s.s.*) and *An. arabiensis* relative to IRS status. In sites with 'no IRS', *An. gambiae* (*s.s.*) and *An. arabiensis* were fairly evenly distributed, in contrast with the predominance of *An. gambiae* (*s.s.*) in 'IRS stopped' sites (apart from Agago) and *An. arabiensis* in 'IRS active' sites. Observed differences in species composition suggested an impact of IRS on malaria vectors, similar to other reports from this region (Musiime et al., 2019). Sustained vector control has previously been associated with changes in *Anopheles* mosquito species composition whereby highly anthropophagic *An. gambiae* (*s.s.*) is replaced by the less anthropophagic *An. arabiensis* (Bayoh et al., 2010; Mwangangi et al., 2013; Mawejje et al., 2021) potentially arising from the tendency of *An. arabiensis* to rest outdoors (Mahande et al., 2007), and behavioral patterns limiting contact with indoor based vector control interventions (Yohannes & Boelee, 2012). Similarly, a study in Tororo (one of the 'IRS active' sites) showed predominant *An. gambiae* (*s.s.*) (up to 77% abundance) prior to IRS, being replaced by *An. arabiensis* after IRS (Musiime et al., 2019). Stopping vector control has been associated with a rebound of primary vector species in some settings (Hargreaves et al., 2000; McCann et al., 2014). Pyrethroid-resistant primary vectors (such as *An. gambiae* (*s.s.*) and *An. funestus*) may have a selective advantage enabling them to overcome pyrethroid-based vector control or less effective non-pyrethroid IRS, resulting in a resurgence of malaria morbidity (Hargreaves et al., 2000). In the 'IRS stopped' district of Agago, in which we recorded predominantly *An. arabiensis*, it is plausible that there were spillover effects from sustained IRS (Namuganga et al., 2021) in the neighboring district of Otuke (Fig. 1), with the 'invasion' of *An. gambiae* (*s.s.*) in this district limited by IRS activity in Otuke. The absence of historical data on species composition pre-vector control implementation in the 'IRS stopped' area, however, limits interpretation of the impact of IRS on malaria vector-species composition. This noted, the consequences of stopping IRS in this region on malaria epidemiology have been associated with a rapid resurgence of the disease to pre-IRS levels (Raouf et al., 2017; Namuganga et al., 2021).

Highly anthropophilic and endophilic mosquitoes (*An. gambiae* (*s.s.*) and *An. funestus*) (Mwangangi et al., 2003) are more likely than zoophilic species (White et al., 1972; Molineaux et al., 1980) to be exposed to LLINs and IRS (Russell et al., 2010). Sympatric populations of *An. gambiae* (*s.s.*) and *An. arabiensis* or *An. funestus*, and zoophilic *An. rivulorum* (Kawada et al., 2012) have often revealed differential levels of mortality to insecticides in *An. gambiae* (*s.s.*) or *An. funestus* compared to *An. arabiensis* (Ochomo et al., 2014) or *An. rivulorum* (Kawada et al., 2012), respectively. In addition, the mechanisms mediating resistance in *An. gambiae* (*s.s.*) and *An. funestus* are more widespread and established (Kawada et al., 2011; Ranson et al., 2011; Mulamba et al., 2014; Ranson & Lissenden, 2016). Here, *An. gambiae* (*s.s.*) was significantly more resistant to pyrethroids than *An. arabiensis,* similar to reports from elsewhere

(Ochomo et al., 2013). The significantly higher levels of pyrethroid resistance observed in *An. gambiae* (*s.s.*) in the 'IRS stopped' sites suggest that halting IRS interventions which have a different target site may open a population to selection by insecticides used for public health and/or agricultural purposes.

This study had several limitations. First, the findings are limited by the cross-sectional sampling done in only 11 districts. This may have introduced bias; however, sampling from several districts provided a snapshot of pyrethroid resistance in geographically distinct areas. Second, the definitions of insecticide resistance are based on WHO cut-offs using diagnostic concentrations of permethrin (0.75%) and deltamethrin (0.05%). Pyrethroid intensity assays to determine the operational significance of insecticide resistance were not conducted due to sample size limitations. Third, sample size limitations may have reduced the statistical power available to adequately test genotype-phenotype associations. Small sample sizes may result in a type II error and failure to reject the null hypothesis, due to an underestimation of the true effect. Nonetheless significant associations between target site/metabolic resistance markers with pyrethroid resistance were found in this analysis. Fourth, the concentration of PBO used was 4.0% which may not be directly comparable to the concentration of PBO on LLINs. In a study of PBO LLINs distributed by the Ugandan Ministry of Health in 2017–2018, the concentration of PBO at baseline was 26.81 g/kg in PermaNet 3.0, and 8.17 g/kg in Olyset Plus (Mechan et al., 2022) which may not be equivalent to the concentration included in the WHO tube assay. Finally, the absence of historical data before LLIN and/or IRS implementation limited the inferences that could be made on the development and spread of pyrethroid resistance mutations. Metabolic resistance mechanisms were not explored in *An. arabiensis* due to resource limitations and a lack of DNA-based markers for assessing metabolic resistance in this species.

5. Conclusions

Resistance to pyrethroids was widespread across Uganda, underscoring the importance of insecticide resistance management strategies targeting both *An. gambiae* (*s.s.*) and *An. arabiensis*. Adding PBO to pyrethroids improved mosquito mortality in both species, supporting the WHO's new recommendation to deploy PBO LLINs for vector control in settings of pyrethroid resistance. Whilst target site resistance marker *Vgsc 995S* seems to be approaching fixation in *An. gambiae* (*s.s.*), the moderate frequency of *Vgsc 995F* in the 'IRS stopped' sites suggests intense insecticide selection pressure in northern Uganda. Our results also suggest an association between metabolic resistance variants (the triple-mutant-*Cyp6p4* and *Cyp4j5*) and *An. gambiae* (*s.s.*) survival following exposure to PBO and pyrethroids underscoring the need for further research on the relationship between markers of metabolic resistance and PBO. Further surveillance of insecticide resistance and assessment of correlations between genotypic markers and phenotypic outcomes are needed to better understand mechanisms of pyrethroid resistance as PBO LLINs are scaled-up and to guide vector control measures.

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Ethical approval

Mosquito collections for this study were approved by the Makerere University College of Health Sciences, School of Medicine research ethics committee (Ref: 2018-066), Uganda National Council of Science and Technology (Ref: SS 4586), and London School of Hygiene and Tropical Medicine Ethics Committee (LSHTM Ethics Ref: 14584) under protocol study title "*Investigating spatial and localized interactions between insecticide resistance, insecticidal malaria vector control and malaria transmission in Anopheles mosquitoes from Uganda*" and by the School of Biomedical Sciences Research and Ethics Committee (Ref: SBS-HDREC-669) and Uganda National Council of Science and Technology (Ref: HS 2629) under study title "*Entomological surveillance of vector behaviour, vector density and insecticide resistance to inform malaria vector control in Uganda*."

CRediT author statement

Henry Ddumba Mawejje, Sarah G. Staedke, David Weetman, Grant Dorsey, Amy Lynd, and Martin James Donnelly: conceptualisation. Henry Ddumba Mawejje, David Weetman, Amy Lynd and Martin James Donnelly: methodology. Henry Ddumba Mawejje, David Weetman, Amy Lynd, Martin James Donnelly: investigation. Henry Ddumba Mawejje, Sarah G. Staedke, Grant Dorsey, Martin James Donnelly, Amy Lynd, Philip J. Rosenthal, Adrienne Epstein, Jonathan Lines, Catherine Maiteki-Sebuguzi, Jimmy Opigo, Moses Kamya, and David Weetman: writing - review & editing. Grant Dorsey, Henry Ddumba Mawejje, Martin James Donnelly and David Weetman: formal analysis. Philip Rosenthal, Moses Kamya, Grant Dorsey, Jimmy Opigo, Catherine Maiteki-Sebuguzi: funding acquisition. Henry Ddumba Mawejje and Sarah G. Staedke: writing - original draft. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Given their role as Co-Editor, David Weetman had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Editor-in-Chief Aneta Kostadinova.

Data availability

The data supporting the conclusions of this article are included within the article and its supplementary files.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2022.100106.

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