Trends in Parasitology

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Review

Genetic surveillance of insecticide resistance in African *Anopheles* populations to inform malaria vector control

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Insecticide resistance in malaria vector populations poses a major threat to malaria control, which relies largely on insecticidal interventions. Contemporary vector-control strategies focus on combatting resistance using multiple insecticides with differing modes of action within the mosquito. However, diverse genetic resistance mechanisms are present in vector populations, and continue to evolve. Knowledge of the spatial distribution of these genetic mechanisms, and how they impact the efficacy of different insecticidal products, is critical to inform intervention deployment decisions. We developed a catalogue of genetic-resistance mechanisms in African malaria vectors that could guide molecular surveillance. We highlight situations where intervention deployment has led to resistance evolution and spread, and identify challenges in understanding and mitigating the epidemiological impacts of resistance.

Highlights

Genetic surveillance to detect mechanisms of insecticide resistance in populations of malaria-transmitting mosquitoes can guide the deployment of new insecticidal vector-control tools that have multiple modes of action within the mosquito.

Novel resistance-associated genetic variants continue to be identified in Africa's major malaria vector species. We develop a catalogue of genetic variants that could be routinely screened in surveillance.

Genetic surveillance can detect rises and spatial spread of resistance following deployment of new insecticidal interventions.

The utility of genetic surveillance in operational malaria control depends on a better understanding of links between resistance genetics, phenotypes, and the efficacy of insecticidal interventions in malaria control. We present evidence demonstrating these relationships, together with sources of current uncertainty.

The need for geospatial information on genetic mechanisms of insecticide resistance

Control of mosquito vectors of malaria using interventions based on chemical insecticides, including insecticide-treated nets (ITNs) and indoor residual spraying (IRS), is critical in suppressing malaria transmission [1]. The development of **insecticide resistance** (**IR**) (see Glossary) in mosquito vector populations threatens to erode the recent successes achieved by these interventions in combatting malaria [2,3]. Resistance to pyrethroids, the class of insecticides most commonly used in vector control, has spread widely throughout populations of major malaria vector species in sub-Saharan Africa since the 1990s [4], including species from the *Anopheles* (*An.*) *gambiae* complex [5] and the *An. funestus* group [6,7]. In several regions, standard pyrethroid-only ITNs now fail to significantly increase mosquito mortality [8–10], and they show lower protection against clinical malaria than newer types of ITNs design to counteract pyrethroid resistance [11–14].

IR in *Anopheles* populations has evolved through a diverse array of underlying genetic mechanisms [15–23] which vary across different classes and types of insecticides [20,24]. Developments in ITN and IRS technology now focus on counteracting multiple different mechanisms of resistance using combinations of partner compounds which have distinct modes of action in the mosquito, to improve toxicity and mitigate selection for IR [25]. Next-generation ITNs, also known as 'new nets', incorporate two compounds, a pyrethroid insecticide combined with either the synergist piperonyl butoxide (PBO) [12,26], or an insecticide which is either pyriproxyfen [27], or the pyrrole chlorfenapyr [28]. These additional compounds vary in their impact on mosquitoes; PBO inhibits the production of enzymes which metabolise pyrethroid insecticides, while pyriproxyfen disrupts mosquito growth and fertility, and chlorfenapyr inhibits mosquito respiration. A wider range of insecticides are available for use in IRS [3]. Newer IRS insecticide products include the

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neonicotinoid clothianidin [29,30] and the novel insecticide broflanilide [31], both of which offer different modes of action compared to other vector-control insecticides. The global plan of the World Health Organisation (WHO) for IR management (IRM) recommends rotating the insecticide classes used in IRS to avoid sustained exposure to a single mode of insecticidal action [25,32], although recent modelling studies suggest that simultaneously applying these multiple insecticides may be a more effective strategy [33].

The design of interventions to mitigate the impacts and spread of resistance needs to be informed by location-specific information about the levels of phenotypic IR in the vector species present, together with the underlying genetic mechanisms. An understanding of how these local resistance profiles affect the efficacy of different vector-control products in combatting malaria is also required to identify regions where particular interventions will be most beneficial (Figure 1). This necessitates the development of quantitative relationships between resistance genetics and phenotypes, vector-control efficacy, and the consequent capacity of insecticide-based interventions to reduce malaria across different epidemiological and environmental settings. These analyses need to be geospatial and location-specific, considering local malaria endemicity, characteristics of the human population and their access to vector control products, as well as vaccines, prophylaxis, and treatment, and ecological factors such as seasonality and vector species composition. Many of these variables are incorporated into the range of mathematical

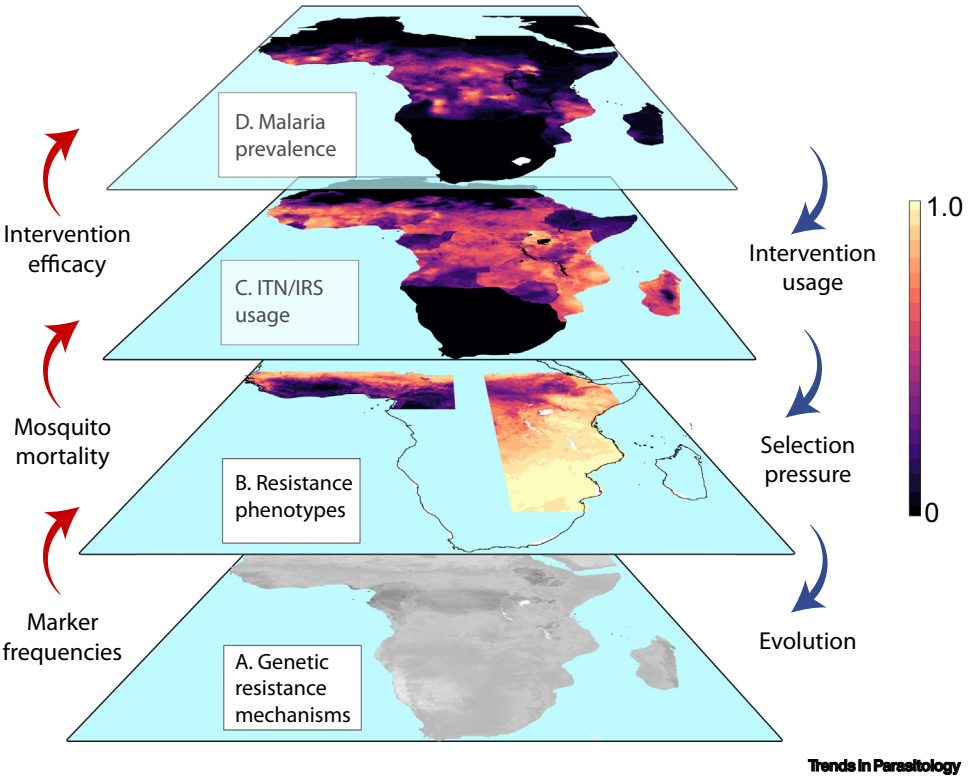

Figure 1. Schematic diagram showing an example of links between the geographic distribution of insecticide resistance (IR) profiles and vector control efficacy. The top three maps show data from predictive maps for the year 2017, including the prevalence of *Plasmodium falciparum* malaria in children aged 2–10 years [35] (top map), ITN usage [117] (second from top), the prevalence of phenotypic resistance to deltamethrin in *Anopheles gambiae* complex populations [5] (third from top). The bottom map is depicted in grey to illustrate the lack of broadscale data on the frequencies of genetic mechanisms of IR.

and statistical models that predict malaria transmission and prevalence as well as intervention efficacy across a range of African settings [3,34,35].

At present, however, comprehensive, contemporary surveillance data on IR is lacking for many African regions. Operational decisions about public health management in sub-Saharan Africa are made at the level of administrative health districts [32]. Resistance phenotypes in mosquito populations are most commonly monitored by standard susceptibility tests [25]; however, the spatial coverage of available data is relatively sparse, with 89% of malaria-endemic administrative districts having no recorded measurements in the period 2015–2017 [5,32]. Data describing genetic resistance mechanisms is sparser still, even for frequencies of pyrethroid target-site resistance, the most widely monitored genetic mechanism. A recent study determined that only nine African countries had sufficient records of the frequencies of pyrethroid target-site resistance in *An. gambiae* complex populations to support geostatistical analyses of geographic variation in resistance frequencies [36].

Encouragingly, capacity for widespread geospatial surveillance of genetic mechanisms of IR is currently expanding. Recent developments in **whole-genome sequencing** (**WGS**) [37,38] have led to rapid growth in genomic datasets from mosquito samples collected throughout malaria endemic countries, while new open resources allow these data to be accessed and analysed by non-specialist users (https://www.sanger.ac.uk/tool/ag1000g/). Genomic analyses have identified new genetic drivers of resistance [19,23,38,39] and given insight into resistance evolution and spread [19,40,41]. WGS is, however, too intensive, and expensive for routine surveillance of vector populations with adequate coverage across administrative districts. For the purposes of high-throughput genetic screening, targeted amplicon-sequencing panels for genotyping a subset of predictive genetic markers of IR [24,42,43], vector species identification [44], and *Plasmodium falciparum* infection [44] are being developed and may represent a feasible tool for expanding field monitoring when combined with advances in low-cost, portable sequencing platforms such as Oxford Nanopore Technology. Moreover, new initiatives to increase the scalability and sustainability of genomic surveillance of vector populations recognise the need to operationalise these resources to contribute to strategic planning by national malaria-control programmes. Here we review current knowledge of the different genetic mechanisms that underly IR in Africa's major malaria vector species, including species from the *An. gambiae* complex and the *An. funestus* subgroup, to inform **genetic surveillance** of resistance and guide intervention deployment decision-making.

Genetic resistance mechanisms in *An. gambiae complex* vector species

The diverse array of genetic IR mechanisms in mosquitoes from the *An. gambiae* complex are typically categorized as either target-site, metabolic, cuticular, or behavioural resistance, with additional categories relating to mechanisms of insecticide sequestration and removal [17]. Here we review evidence of the genetic basis for these mechanisms with the aim of summarizing the known genetic variants that could be screened as part of molecular surveillance programmes, addressing current data gaps (illustrated in Figure 1A). For each resistance mechanism, the SNP mutations showing associations with resistance are summarized in Table 1.

Target-site resistance

The insecticides that are currently recommended by the WHO to control adult malaria vectors fall within seven classes: carbamates, organochlorines, organophosphates, neonicotinoids, pyrethroids, pyrroles, and insect growth regulator antagonists. The insecticide types used in IRS span all these classes except for pyrrole and growth regulator insecticides and includes the novel *meta*-diamide broflanilide. For ITNs, only pyrethroids, or a combination of pyrethroid

Glossary

Copy number variant (CNV): a genomic variant whereby a sequence of nucleotides is repeated in tandem multiple times in an individual's genome.
Cross-resistance: resistance to one insecticide by a mechanism that also confers resistance to another insecticide, even when the insect population has not been selected by exposure to the latter.
Diagnostic dose: the amount of insecticide active ingredient that mosquitoes are expected to absorb while being exposed to a discriminating concentration of an insecticide for a fixed period, and which reliably kills all susceptible mosquitoes.
Discriminating concentration: the concentration of an insecticide that, when mosquitoes are exposed to it for a standard period, reliably kills all susceptible mosquitoes.
Genetic surveillance: widespread screening of wild mosquito populations for genetic variants associated with IR, using a suite of conventional molecular assays.
Genome-wide association study (GWAS): an observational study used to identify genomic variants that are statistically associated with a particular trait.
Insecticide resistance (IR): a heritable change in the sensitivity of an insect population which may lead to survival following exposure to a standard dose of insecticide. This may be the result of physiological or behavioural adaptation.
Phenotypic resistance: development of an ability in a subpopulation of insects to tolerate doses of insecticides that would prove lethal to most insects in a susceptible population of the same species.
Resistance intensity: the strength of IR in mosquitoes, resulting from the level of expression of resistance phenotype(s).
Susceptible population: a mosquito population is susceptible to an insecticide when 98% or more of the population die after being exposed to a discriminating concentration of the insecticide.
Whole-genome sequencing (WGS): the process of elucidating the entire DNA sequence of an organism's genome.

Table 1. Genetic markers that show associations with IR phenotypes in the *An. gambiae* complex

Mechanism	Susceptible allele	Resistance alleles	Insecticide types	Refs
Target site				
Voltage-gated sodium channel	*Vgsc*-402V	*Vgsc*-402L	Type I and II pyrethroids, DDT	[19,63]
Voltage-gated sodium channel	*Vgsc*-995L	*Vgsc*-995F, *Vgsc*-995S,	Type I and II pyrethroids, DDT	[55,56,61]
Voltage-gated sodium channel	*Vgsc*-1570N	*Vgsc*-1570Y	Type I and II pyrethroids, DDT	[62]
Voltage-gated sodium channel	*Vgsc*-1527I	*Vgsc*-1527T	Deltamethrin, Permethrin	[38]
Gaba receptor	*Rdl*-296C	*Rdl*-296G, *Rdl*-296S	Organochlorines	[48]
Acetylcholinesterase insensitivity	*Ace1*-280G	*Ace1*-280S	Carbamates, Organophosphates	[38,64–66, 70,115]
Metabolic				
Glutathione S-transferase overexpression	*Gste2*-114I	*Gste2*-114T	DDT	[24,69]
Glutathione S-transferase overexpression	*Gste2*-119L	*Gste2*-119V	Permethrin	[24]
Cytochrome P450 overexpression	*Cyp4j5*-43L	*Cyp4j5*-43F	Permethrin, Deltamethrin	[38,67]
Carboxylesterase overexpression	*Coeae1d*-T	*Coeae1d*-C	Permethrin	[67]
Cytochrome P450 overexpression	*Cyp6p4*—236M-*Cyp6aap*	*Cyp6p4*-236M-*TE-Cyp6aap*-Dup1	Deltamethrin	[39]

and either the synergist PBO, the pyrrole chlorfenapyr, or the insect growth regulator piriproxyfen, are incorporated into the set of ITN products that have attained WHO pre-qualification status. The physiological target of the insecticide within the mosquito varies across these different insecticide types and classes, and therefore the genetic basis of target-site resistance also differs. Except for pyrrole insecticides and insect growth regulator antagonists, all other vector-control insecticides are neurotoxic. Dichlorodiphenyltrichloroethane (DDT) and pyrethroid insecticides target the voltage-gated sodium channel (Vgsc) [45], and the organochlorine dieldrin and *meta*-diamide broflanilide target the Gaba receptor, a chloride channel [46–48]. Organophosphate and carbamate insecticides inhibit acetylcholinesterase (AChE), an enzyme that is essential to the control of neuronal signalling [49,50]. The neonicotinoid clothianidin overstimulates and blocks nicotinic acetylcholine receptors (nAChR), leading to paralysis and death [30]. The pyrrole chlorfenapyr utilises a different mode of action, interfering with oxidative phosphorylation in the mitochondria, causing death by depriving the organism of energy [51,52]. The growth regulator pyriproxyfen is a juvenile hormone analogue that also offers a unique mode of action, inhibiting arthropod morphogenesis, reproduction, and embryogenesis of insects [53]. In *Anopheles* mosquito species it has been shown to cause sterility in adult females and shorten their lifespan [54].

In vector species from the *An. gambiae* complex, genetic mechanisms conferring target-site resistance within genes encoding the Vgsc [19,55,56], the Gaba receptor (Figure 2) [46], and AchE [49], have become widespread [23,24,36,37], resulting in **phenotypic resistance** to pyrethroids, organochlorines, organophosphates and carbamates (Figure 2). For these forms of target-site resistance, several genetic mutations that show associations with resistance phenotypes have been identified (Table 1). Target site resistance mechanisms have not yet been identified for chlorfenapyr in *An. gambiae* complex species, although chlorfenapyr resistance has evolved in other insect species and the genetic basis has been studied [52]. Recently, phenotypic resistance to chlorfenapyr has been reported in *An. gambiae* populations in the Democratic Republic of the Congo (DRC), Cameroon, and Ghana [57]. *An. gambiae* complex mosquitoes remain largely susceptible to clothianidin [58], although strong phenotypic resistance to this insecticide was found in some *An. gambiae* populations in Cameroon [29], with upregulation of cytochrome P450 enzymes indicating underlying metabolic resistance mechanisms. It is also likely that P450-mediated metabolic resistance may cause resistance to pyriproxyfen [59].

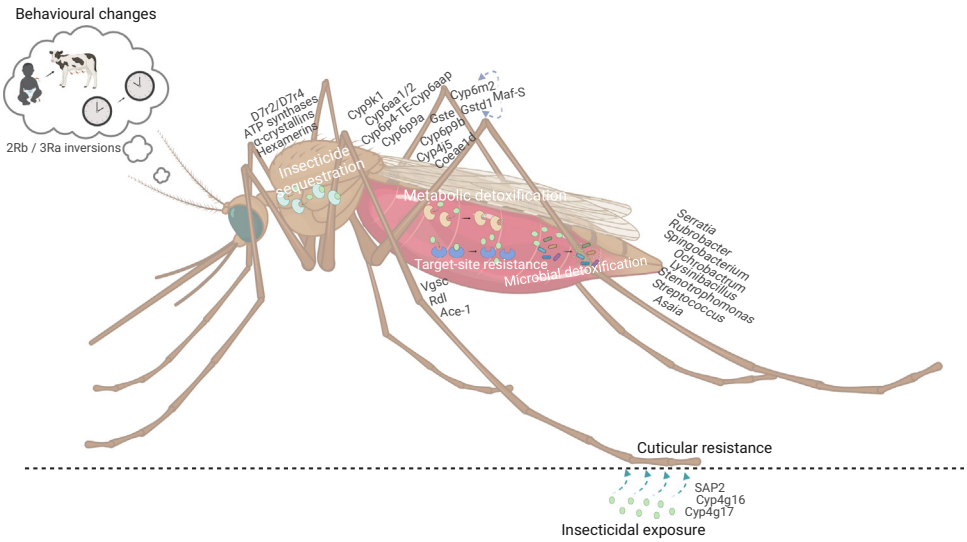

Figure 2. Multiple insecticide resistance (IR) mechanisms linked to IR phenotype in *Anopheles gambiae*. Current IR mechanisms in malaria vector species may be broadly classified into six categories: target-site resistance; metabolic detoxification; insecticide sequestration; microbial detoxification, cuticular resistance; and behavioural changes. Implicated genes/proteins/biological markers involved per IR mechanism group are detailed.

In the case of pyrethroid resistance, characterisation and validation of SNPs conferring target-site resistance in the *An. gambiae* complex dates to the 1990s when two SNPs at the same locus in the *Vgsc* gene, *Vgsc*-995F and *Vgsc*-995S, were found to confer 'knock-down' (*kdr*) resistance to pyrethroids and DDT (Table 1) [55,56,60]. These two SNPs are commonly referred to as the west and east African *kdr* mutations because *Vgsc*-995F was first detected in west Africa [55], and is predominant there, while *Vgsc*-995S was more commonly reported in the east. Both mutations occur throughout the east and west now; and they can co-occur in individual mosquitoes, which is common in parts of central Africa such as the DRC [36]. For both mutations, genotype-phenotype associations have been repeatedly demonstrated using a range of approaches (for a review see [60]). In the case of *Vgsc*-995F, a recent study used clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 gene-editing techniques to establish associations with resistance to pyrethroids and DDT by comparing mosquitoes with susceptible and resistant alleles against the same genetic background [61], provided clear functional validation of the role of this mutation in conferring resistance.

Genomic studies have identified several other mutations in the *Vgsc* that could potentially contribute to IR, highlighting the complex genetic basis of target-site resistance in the *An. gambiae* complex [19]. For some of these mutations, associations with resistance phenotypes have been demonstrated (Table 1). The *Vgsc*-N1570Y mutation has been shown to intensify resistance to DDT and pyrethroids and is linked with the *Vgsc*-995F genotype [62]. The *Vgsc*-402L mutation [19] has been implicated in resistance to pyrethroids and DDT [63], and it confers a lower fitness cost on the mosquito compared to *Vgsc*-995F under laboratory conditions, indicating a potential selective advantage to this resistance mechanism.

While *Vgsc* resistance mechanisms have received the most extensive study, mutations in other insecticide target sites that confer resistance to other insecticide types and classes have been identified in the *An. gambiae* complex (Table 1 and Figure 2). Two SNPs in the 'resistance to dieldrin' (*Rdl*) locus that encodes a Gaba receptor subunit decrease susceptibility to dieldrin [48]. Despite

the ban on use of such cyclodienes in 2001 by the Stockholm Convention on Persistent Organic Pollutants, due to slow environmental degradation, *Rdl* mutations have persisted decades later in some malaria vector populations [46]. Resistance to organophosphate and carbamate insecticides is conferred by a SNP in the *Ace-1* gene (G119S) that encodes for AChE causes insensitivity of the enzyme [49,64,65] (Table 1 and Figure 2). This mutation incurs a high fitness cost, but duplications have arisen whereby susceptible and resistant alleles occur on the same chromosome [66], potentially reducing the fitness cost of this resistance mechanism [65,66].

Metabolic resistance

Ranson *et al*. [15] define metabolic resistance as 'the overexpression of enzymes capable of detoxifying or sequestering insecticides and/or amino acid substitutions within these enzymes which alter the affinity of the enzyme for the insecticide'. Enzymes that detoxify insecticides include certain cytochrome P450s, glutathione-*S*-transferases and carboxylesterases [15,20]. In comparison to target-site resistance, it has proven more difficult to identify genetic markers associated with metabolic resistance [15,20,67,68]. Changes to metabolic gene expression are conventionally assessed using qRT-PCR (quantitative real-time PCR) assays; however, the cold-chain requirements to measure metabolic gene overexpression represents a barrier to routine surveillance of these resistance mechanisms in resource poor settings. Encouragingly, recent studies have made new discoveries of mutations within genes encoding detoxifying enzymes that show associations with resistance phenotypes in *An. gambiae* complex members (Table 1). A SNP in *Cyp4j5*, a cytochrome P450 gene, was found to be significantly associated with resistance to permethrin in *An. gambiae* (Table 1; [67]) and a SNP in *Coeae1d*, a carboxylesterase gene, was also significantly associated (Table 1 and Figure 2; [67]). Njoroge *et al*. [39] identified a haplotype containing three mutations, including a SNP in *Cyp6p4*, a transposable element insertion, and a duplication of the *Cyp6aa1* gene (Table 1). The first two of these mutations are in tight linkage, with haplotypes containing only these two mutations being referred to as double mutants, and haplotypes containing all three mutations being referred to as triple mutants [39]. The triple mutant haplotype was found to be strongly associated with resistance to deltamethrin and has spread rapidly in *An. gambiae s.s.* populations in Uganda, Kenya and the DRC. The triple mutant is predominant, having replaced the double-mutant haplotype, suggesting a strong selective advantage [39]. These findings add to the catalogue of genetic markers of metabolic resistance in *An. gambiae* complex species. Previously, the only known DNA markers of metabolic resistance were the SNPs in a glutathione-S-transferase gene, *Gste2*-114T and *Gste2*-119V (Table 1 and Figure 2), which were found to confer resistance to DDT and permethrin in *An. gambiae* s.s., respectively [24,69].

There is considerable uncertainty about the mechanisms underlying metabolic resistance and the extent of their contribution to resistance phenotypes [68,70,71]. In West Africa intense phenotypic pyrethroid resistance (>1500-fold compared to a **susceptible population**) has been reported from *An. gambiae* complex populations, with more modest overexpression of key cytochrome P450s [72]. Synergistic effects of P450-mediated resistance and target-site mutations have been reported [68,70], but the individual contribution of metabolic factors is unclear, and may not to be linked to the extent of upregulation [68].

Gene expression can also be elevated by increases in copy number of genes (**copy number variants; CNVs**) [23]. WGS analysis has revealed the importance of CNVs to IR, with a high proportion of the genes that have been linked to IR having demonstrable CNVs [23]. Current widescale CNV detection is based on qPCR assays, notably to estimate *Cyp6aa/Cyp6p* duplications [39]. Elucidating the relative contribution of each CNV to IR is more challenging [23]. Across West Africa, CNVs have been identified in *Cyp6aa* and *Gste2* at high frequencies in *An. coluzzii*

populations, which were associated with deltamethrin resistance [38]. The gene clusters showing CNVs are variable across different vector species, and subpopulations of a given species, complicating detection and surveillance of these resistance mechanisms [38].

Regulation of particular metabolic genes, including *Cyp6m2* and *Gstd1*, has been linked to expression levels of the transcription factor Maf-S; RNAi-attenuation of Maf-S significantly increased mortality to DDT, permethrin and deltamethrin and decreased mortality to malathion, providing a mechanistic explanation for negative **cross-resistance** between certain pyrethroids and organophosphates [16]. There is evidence, however, that some cytochrome P450 activity may be regulated by distant loci rather than directly by local variants [20,71,73]. The predictive value of DNA markers in capturing the phenotype contribution of metabolic resistance is therefore uncertain [20,68].

Other resistance mechanisms

The complexity of IR is increasingly being demonstrated, with studies finding potentially extensive polygenicity in resistance mechanisms [16,17,19,23,24,37,74], and heterogeneity in genetic mechanisms across species and geographic locations [16–18,36–38]. In addition to target-site and metabolic resistance, there are others, with recent studies revealing a widening array of mechanisms operating through different biological functions within the mosquito (Figure 2). Cuticular resistance, whereby mosquitoes resist insecticides through thickening of the epicuticle layer and greater cuticular hydrocarbon (CHC) content [21,22] has been associated with increased expression of two cytochrome P450 genes, *Cyp4g16* and *Cyp4g17* [21,68]. Recently, ABC transporters, proteins that mediate efflux of foreign compounds from cells, were found to be differentially expressed in pyrethroid resistant *An. gambiae* mosquitoes and enriched in the legs [75]. Similarly, hexamerins and α-crystallins, which play binding and storage roles, have been implicated in pyrethroid resistance and may function by sequestering insecticides [17]. Other gene families involved in transportation, including salivary proteins (e.g., D7r4), are also upregulated in resistant *An. gambiae*, resulting in increased pyrethroid resistance via insecticide sequestration, rather than direct detoxification [17,74,76]. Expression of a chemosensory protein (SAP2), enriched in the head and legs of *An. gambiae* has been shown to mediate pyrethroid resistance by binding at point of insecticide contact [77]. In *An. coluzzii,* resistant mosquitoes display increased respiration through changes in entire metabolic pathways, linked to genes involved in oxidative phosphorylation [16]. In addition to host-mediated resistance mechanisms, evidence is emerging that alterations to the mosquito microbiota can also contribute to IR, likely via endosymbiont degradation of insecticides [78]. Unfortunately, there are currently no known DNA markers for these variety of IR mechanisms beyond target-site and metabolic resistance.

Other knowledge gaps present a challenge to developing a full understanding of genetic resistance mechanism in malaria vector species. Shifts in mosquito behaviour that allow them to avoid contact with insecticides, for example by biting humans outdoors and/or during day or evening hours, has become a major problem contributing to the declining efficacy of indoor insecticidal interventions, including ITNs and IRS [79–81]. A genetic component associated with host choice in *An. arabiensis* may involve paracentromeric inversions of chromosomes 2Rb and/or 3RA; however, the heritable mechanisms of these behavioural changes require further investigation [82]. Interestingly, the 2Rb inversion has also shows an association with insecticide resistance in *An. coluzzii* [16], indicating that chromosomal inversions are another resistance mechanism that could potentially inform genetic surveillance.

Genetic resistance mechanisms and associated markers in the *An. funestus* group

Compared to *An. gambiae,* the genetic resistance mechanisms present in the *An. funestus* group are less clearly defined. A SNP at N485I in *Ace-1* has been found to be associated with

carbamate resistance [83], and a point mutation in the *Rdl* locus that encodes the Gaba receptor subunit, also shared with *An. gambiae*, confers resistance to dieldrin [46]. More recently *Vgsc*-976F (equivalent to *Vgsc*-1014F in *An. gambiae*) was identified, in tight linkage disequilibrium with *Vgsc*-1842S, in *An. funestus* which had higher survivorship to DDT [84] (Table 2). Most studies on *An. funestus* indicate that metabolic overexpression of cytochrome P450s drives IR in this vector species. A mutation in the gene *Gste2* (*Gste2*-L119F) has been implicated in resistance to DDT, pyrethroids and neonicotinoids ([73,85]; Table 2). Several cytochrome P450 genes, including *Cyp6z1* [83], *Cyp6p4a* [86], *Cyp6*n1 [87], *Cyp9k1* [88], and *Cyp6*m7 [89] are commonly overexpressed in resistant *An. funestus* populations. Overexpression of *Cyp6p9a* and *Cyp6p9b*, mediated by structural changes to transcription factor binding sites (*cis*-regulatory elements), also plays a key role in pyrethroid resistance and can result in reduced bioefficacy of ITNs ([9,86,89,90]; Table 2). Patterns of cytochrome P450 overexpression may be broadly associated with geography, with different complements of genes upregulated among distinct resistant *An. funestus* vector populations in west and east Africa (with *Cyp6p9a, Cyp6p9b*, and *Gste2* predominating in the west, and *Cyp6m4, Cyp9k1, Cyp6m7, Cyp6n1, Cyp6m1*, and *Cyp6z1* in the east) recently reviewed [91]. The prevalence of alternate IR mechanisms present in *An. funestus* is largely unknown due to a relative paucity of exploratory datasets, by comparison to *An. gambiae.*

Characterising resistance phenotypes

To determine functional associations between IR mechanisms and phenotypic resistance (Figure 1B), it is essential to first characterise mosquito phenotype prior to performing a **genome-wide association study** (**GWAS**). Phenotypic IR among *Anopheles* vector populations is conventionally established using standard bioassays [92] which measure mosquito mortality following exposure inside plastic tubes to filter papers containing a **discriminating concentration** (**DC**) of insecticide or glass bottles coated with a **diagnostic dose** (**DD**) of insecticide. This approach to quantifying phenotypes faces difficulties with high test variability and non-standard outcomes across assays and may not provide measures that are operationally relevant to the efficacy of insecticidal vector control tools in field settings (Box 1).

Spatiotemporal trends in IR

IR is a dynamic phenomenon that can evolve rapidly in natural mosquito populations, particularly in response to environmental selection pressures (Figure 1). Rapid increases in resistance phenotypes and genetic mechanisms associated with the widespread implementation of ITN interventions in sub-Saharan Africa since the early 2000s [93] have been observed. In the case of pyrethroid target site mutations, haplotype diversity analyses have shown evidence of selective sweeps in both the *Vgsc*-995F and *Vgsc*-995S mutations in mosquitoes from west and east

Table 2. Genetic markers that show associations with IR phenotypes in the *An. funestus* group

Mechanism	Susceptible allele	Resistance alleles	Insecticide types	Refs
Target site				
Acetylcholinesterase insensitivity	*Ace-1*-485N	*Ace-1*-485I	Carbamates	[83]
Gaba receptor	*Rdl*-A296	*Rdl*-296S	Organochlorines	[46]
Voltage-gated sodium channel	*Vgsc*-976L + *Vgsc*-1842P	*Vgsc*-976F + *Vgsc*-1842S	DDT	[84]
Metabolic				
Cytochrome P450 overexpression	*Cyp6p9a*	Presence of CCAAT box and CnCC/MafK binding sites	Pyrethroids	[90]
Cytochrome P450 overexpression	*Cyp6p9b*	Presence of CCAAT box, CncC nrf2/MAF binding sites	Pyrethroids	[9]
Glutathione S-transferase overexpression	*Gste2*-119L	*Gste2*-119F	DDT, Permethrin	[116]

Box 1. Challenges in characterising susceptibility to novel insecticides

- Standardised WHO and CDC IR bioassays have issues with direct quantitative comparability between outcome measurements due to differences in insecticide doses used, diagnostic exposure and holding times.
- Testing methodologies for novel, slow-acting insecticides, including clothianidin and chlorfenapyr and non-neurotoxic chemicals (such as pyriproxyfen), cannot rely on the same end-point mortality outcomes used for other public health insecticides.
- Differences in rearing conditions of insectary *Anopheles* colony strains, including larval conditions (e.g. crowding, access to nutrition etc.), time of testing (e.g., night or day), temperature and humidity, mosquito age, and physiological stage can all influence observed bioassay mortality [110], and therefore constitute a source of variability when establishing new DDs.
- Long-term maintenance of insectary colonies in independent testing facilities can also lead to differences in relative colony fitness, following genetic divergence over time, with several studies reporting discordant bioassay data using the same strains, advocating for periodic in-depth strain characterisation at both phenotypic and genotypic levels [111,112].
- All of these factors need to be considered during DD discovery, to differentiate between detection of incipient resistance to novel insecticides, rather than pre-existing natural tolerance among wild vector populations [57,113].
- While phenotypic bioassays are crucial to distinguish insecticide resistant individuals per population for downstream genetic analysis, one final limitation is how vector survival in a bioassay relates to intervention operational efficacy. For example, exposure of mosquito strains classified as resistant to the DC of deltamethrin in WHO tube tests exhibited >90% mortality over 24 h following deltamethrin ITN contact, complicating the functional consequences of vector populations demonstrating IR in bioassays [114].

Africa, which were supported by an observed rapid increase in the frequency of the *Vgsc*-995F mutation in *An. coluzzii* populations in Ghana over a period of approximately 100 generations from 2002 to 2011 [40,94]. Similarly, a strong rise in *Vgsc*-995F frequencies was seen in *An. coluzzii* populations in Burkina Faso from 2000 to 2006 [95]. The spread of *Vgsc-995F* in *An. coluzzii* has resulted from adaptive introgression from *An. gambiae* through hybridisation between the two sibling species [96], with WGS showing that this has involved transfer of a large genomic region surrounding the *Vgsc* gene from *An. gambiae to An. coluzzii* [40]. Temporal increases in frequencies of *Vgsc*-995S in east Africa have also been observed, with a study in Burundi showing a rise in frequency in *An. gambiae s.l.* from around 1% to as high as 86% from 2002 to 2007 in association with an upscaling of IRS and ITN interventions [97]. While the *Vgsc*-995F mutation has historically been uncommon in east Africa [36], it was detected in western Kenya in 2014 [98]. Recent surveillance data collected throughout a large region of Uganda as part of a cluster randomised controlled trial (CRCT) to assess the epidemiological impacts of a rollout of 'new nets' showed spatial spread and propagation of *Vgsc*-995F in western parts of Uganda, where it is thought to have migrated from neighbouring regions in the DRC [99].

Increases in metabolic resistance mechanisms have also been seen in wild *Anopheles* populations. In *An. funestus* populations from southern Africa (Malawi and Mozambique), WGS analyses revealed that *Cyp6p9a* has swept to fixation during the period post-ITN deployment throughout the region, but this did not occur in *An. funestus* populations from more northern areas [6]. In *An. gambiae* from Bioko Island, two cytochrome P450 genes, *Cyp9k1* and *Cyp6p3*, showed increased expression over the period 2011–2015 during which both pyrethroid-based IRS and ITN usage was scaled up [100]. In *An. gambiae* populations from areas of Uganda, Kenya and the DRC, WGS analyses identified a haplotype involving a trio of mutations in the *Cyp6aa/Cyp6p* genomic region which has swept to near fixation over the period 2008–2018 and is strongly associated with resistance to deltamethrin [39]. In eastern regions of Uganda where this 'triple mutant' was uncommon in 2018, further increases in frequency have been observed following a rollout of 'new net' interventions [99].

Many of the insecticides used in malaria vector control are the same as those used in agricultural practices throughout sub-Saharan Africa, including insecticides from the pyrethroid, neonicotinoid, organophosphate and carbamate classes. The use of insecticides in agriculture has been associated

with higher levels of IR in malaria vector populations. A meta-analysis evaluating the relationship between agricultural insecticide use and IR found that 23 out of 25 studies reported an association [101]. More recent studies have found high and increasing levels of resistance to pyrethroids, carbamates and DDT, and moderate resistance to malathion, in agricultural areas of sub-Saharan Africa. In these areas, usage of agricultural pesticides was found to be poorly controlled, and there was a limited awareness of the issue of IR amongst local communities [102,103].

Detecting trends in resistance through geospatial surveillance

The aforementioned illustrations of the complex dynamics of IR highlight the need for continued surveillance of resistance in malaria vector populations. IR can be highly spatially heterogeneous, and an expansive spatial coverage of surveillance data is needed to accurately quantify trends in resistance. For example, marked differences in the frequencies of different genetic resistance mechanisms were found between *An. gambiae* complex species sampled from different places within south-west Burkina Faso [18]. Strategic deployment of vector control interventions including new types of ITNs and IRS requires knowledge of resistance profiles across all administrative planning units. Geostatistical models fitted to surveillance data on resistance phenotypes [5] and genetic mechanisms [36] have demonstrated good accuracy in spatial extrapolation across data sparse regions. These models have been used to generate predictive maps of resistance which can provide estimates in areas that are not well surveyed (Figure 1B). Moreover, model-based approaches can identify underlying trends in noisy surveillance data and have revealed patterns of association between different types of phenotypic resistance [104], and between phenotypes and genetic mechanisms [36].

The genetic surveillance of IR conducted throughout the Uganda LLINEUP trial provides an encouraging example of the value of longitudinal surveillance with comprehensive spatial coverage in revealing trends [99]. The study demonstrated increases and spatiotemporal expansion in three genetic mutations associated with pyrethroid resistance following a nationwide rollout of different types of 'new nets'. We used the time series of fine-resolution predictive maps produced by Lynd *et al*. 2024 [99] to develop estimates of the average rate of increase in the frequency of *Vgsc*-995F for each of the level two administrative planning units in Uganda (Figure 3). This highlights areas in the northwest where this form of resistance is spreading quickly, as well as neighbouring areas that are at high risk of further increases and warns that a decline in vector control efficacy may be expected in these areas.

Investigating links between IR and malaria vector control

Understanding the impacts of IR on the efficacy of insecticidal vector-control interventions in controlling malaria is clearly critical in allowing knowledge and surveillance of resistance to inform intervention deployment (Figure 1D). These relationships have, however, proven difficult to quantify [105,106]. Nonetheless, recent results from CRCTs of different types of 'new nets' indicate that ITN efficacy is improved when they incorporate insecticide combinations among resistant vector populations. Two CRCTs conducted in Tanzania [12] and Uganda [14] both found that ITNs incorporating a pyrethroid (either deltamethrin or permethrin) plus PBO were significantly more effective in reducing malaria prevalence than standard pyrethroid-only ITNs. Two CRCTs conducted in Tanzania [11] and Benin [13] found that ITNs incorporating the pyrethroid alpha-cypermethrin plus chlorfenapyr showed substantially higher efficacy, however the first of these trials found the efficacy of pyrethroid-PBO nets to be similar to pyrethroid-only nets [11]. These trials also assessed 'new nets' incorporating alpha-cypermethrin and pyriproxyfen and found that their efficacy was similar to pyrethroid-only ITNs [11,13]. However, an earlier CRCT conducted in Burkina Faso did find that nets containing pyriproxyfen gave more protection against malaria, compared to pyrethroid-only ITNs [27].

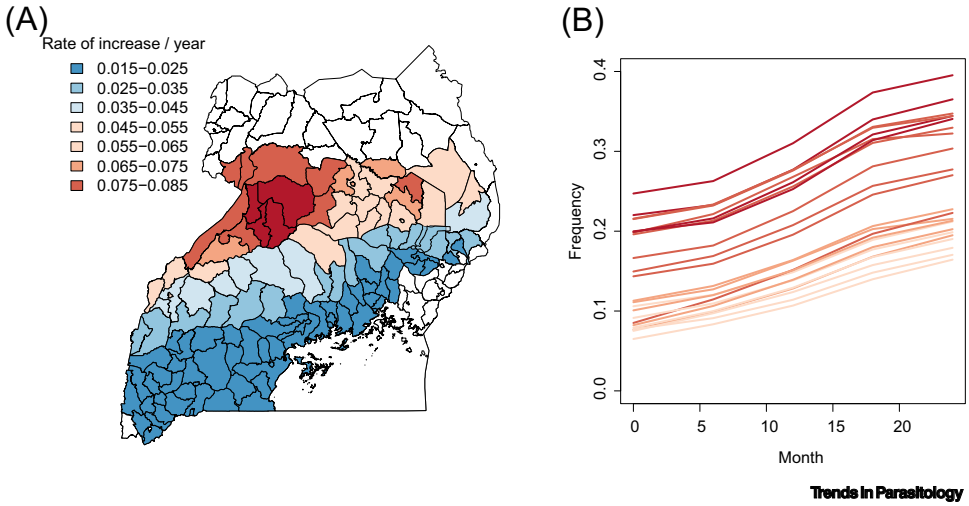

Figure 3. Increases in the frequency and spatial spread of *Vgsc-995F* in Uganda following deployment of insecticide-treated bednets. (A) The predicted average annual rate of increase in the frequency of *Vgsc*-995F in *Anopheles gambiae* populations over the duration of the Uganda LLINEUP study (2017–2019) in each level two administrative area. Rates of increase for each administrative area are the average across all pixels in each area of the mean values of fine-resolution predictive maps [99]. (B) Frequencies of *Vgsc*-995F in *An. gambiae* over time for the top 15% of administrative areas with the highest average annual rates of increase (A).

Experimental hut trials (EHTs) examining the efficacy of ITNs on personal protection demonstrated the effects of IR in reducing mortality of host-seeking female mosquitoes and increasing blood-feeding rates. A meta-analysis of 34 EHTs where volunteers slept under pyrethroid-only ITNs found that phenotypic pyrethroid resistance, as measured by standard susceptibility tests, was associated with higher survival rates among mosquitoes entering experimental huts [107]. Surviving mosquitoes also had a greater probability of successfully blood feeding [107]. Similar trends have also been found in EHTs assessing pyrethroid IRS insecticides [108]. EHTs have also implicated genetic mechanisms of resistance in reducing ITN efficacy. In *An. funestus*, SNPs in the metabolic resistance genes *Gste2, Cyp6p9a* and *Cyp6p9b* have been associated with greater blood feeding success [89,90,109] and higher survival [89,90] in EHTs.

Concluding remarks

Despite the open questions surrounding the epidemiological implications of IR (see Outstanding questions), the observed increases and spread of multiple types of resistance following the upscaling of insecticide-based vector control interventions is highly concerning [87] (e.g., Figure 3). African malaria vectors are still largely susceptible to newer vector-control insecticides, including chlorfenapyr, clothianidin, and broflanilide [58], and the longevity of new ITN and IRS products depends on our ability to prevent vectors from developing resistance to these different insecticide modes of action. Here we have provided information on a catalogue of markers that can potentially be incorporated into routine screening to detect local rates of increase and spatial spread of important resistance mechanisms, serving as an early warning system for geographically contiguous regions. This is only a partial representation of the resistance profiles present in local vector populations, and continuing advances in WGS analyses will be invaluable in developing improved strategies for genetic surveillance going forward. Surveillance of resistance phenotypes remains important given the uncertainty surrounding the influence of genetic mechanisms on the phenotype, and guidelines for intervention deployment need to be based on both genetic and phenotypic information [32]. While not covered in our review, other vector species also play an important role in malaria transmission in Africa, including other species from the *An. gambiae* complex as well as the invasive

Outstanding questions

How well can the set of known insecticide resistance-associated genetic variants occurring in different malaria vector species predict the operational efficacy of current ITN and IRS products in controlling malaria?

How specific are insecticide resistance mechanisms to individual insecticides or insecticide classes, and what is the likelihood of selecting for cross-resistance mechanisms following intervention deployment?

What insecticide resistance management (IRM) strategies will effectively mitigate against the spread of resistance that follows the upscaling of insecticidal interventions? What IRM strategies will be pragmatic for control programmes that are limited in their capacity for resistance surveillance and their ability to deploy new tools in the face of rising resistance?

What capacities and resources can be developed to allow national malaria control programmes to screen for an array of resistance-associated genetic variants in routine surveillance?

Can genomic methods enable quantification of the **resistance intensity** of metabolic resistance in field vector populations as part of routine surveillance?

Can genetic surveillance detect the early emergence of novel insecticide resistance mechanisms in vector populations?

Do frequencies of resistance-associated genetic variants in vector species populations show predictable spatiotemporal relationships that can be reliably extrapolated across unsampled geographic areas? Can these trends provide early warning of the spatial spread of resistance across connected regions?

urban malaria vector *An. stephensi*. Relatively little is known about genetic IR mechanisms in these species, which is a barrier to developing strategies for resistance management and surveillance that account for the variety of species that comprise malaria vector populations.

The costs and resources associated with resistance surveillance present a significant challenge to its scalability and incorporation into national malaria control programmes. GWAS and phenotypic studies to validate GWAS hits are an order of magnitude more expensive than routine molecular surveillance, meaning that detailed characterisations of local resistance mechanisms cannot typically inform timely, responsive decision making. Moreover, with increasing GWAS studies, the number of putative SNPs and other genetic variants is set to increase, thus research efforts must also be dedicated to functional validation of resistance-associated loci to optimise the design of marker panels for genetic surveillance. The procurement of new net and IRS products is also inevitably financially constrained, restricting the areas that can receive new interventions that are available for resistance management (see Outstanding questions). Given the limited capacity of national control programmes to respond rapidly to increasing resistance, pragmatic evidence-based resistance management strategies are needed to protect the longevity of the cadre of currently effective insecticides.

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Declaration of interests

The authors declare no competing interests.

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