



# Pyrethroid resistance mechanisms in the major malaria vector species complex

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With 1 figure

**Abstract:** Pyrethroids remain the most important class of insecticides for controlling malaria mosquitoes. Resistance to pyrethroids in the major African malaria vector *Anopheles gambiae* was detected soon after the introduction of this insecticide class in the 1970s but the strength, and distribution, of this resistance has greatly accelerated in the 21<sup>st</sup> century. In this review we summarise the current understanding of the mechanisms underpinning this resistance, including new discoveries on the genetic basis of established mechanisms such as changes in the neuronal target site of pyrethroids, and latest understandings on less well characterized mechanisms such as insecticide sequestration. Many gaps remain in our understanding of the genetic pathways controlling these resistance associated genes, and for many, the causal resistance mutations remain elusive; this is a key obstacle in the development of informative panels of genetic markers that would aid in the monitoring and management of insecticide resistance in malaria vectors.

**Keywords:** *Anopheles gambiae*, insecticide resistance, insecticide treated nets, resistance mechanisms

## 1 Introduction

Malaria, caused by the parasite *Plasmodium* and transmitted by *Anopheles* mosquitoes remains one of the worlds deadliest diseases with over 600 000 deaths in 2020 alone (WHO 2021). The majority of the malaria burden falls across the African continent, with children under 5 the most vulnerable to this disease (WHO 2021). As the use of insecticides intensified in the 21<sup>st</sup> century, mainly in the form of insecticide treated bednets (ITNs) and, to a lesser extent, indoor residual spraying (IRS), malaria cases and deaths significantly decreased. Indeed, between the years 2000 and 2015, over 80% of the reductions in malaria case numbers could be directly attributable to these interventions (Bhatt et al. 2015). However, since 2015 the gains in malaria control have plateaued, due at least in part to insecticide resistance in major malaria vectors (Churcher et al. 2016).

Until 2020 pyrethroids were the only insecticide found in distributed bednets. More recently new classes of ITNs, containing either the synergist piperonyl butoxide (PBO) (Staedke et al. 2020), the insect growth regulator pyriproxyfen (Ngufor et al. 2014) or the pyrrole insecticide chlorfenapyr (Bayili et al. 2017) have become available. At the time

of writing, only the pyrethroid-PBO nets had a WHO policy recommendation; these nets now make up approximately 50 % of the nets procured for use in Africa and the number of orders for other, dual active nets is steadily increasing in anticipation of a WHO policy recommendation later in 2022. Despite the availability of these new active ingredients, all ITNs still rely on the fast-acting pyrethroids for the rapid knockdown that provides the personal protection to bed net users. Hence the rapid increase in resistance to pyrethroids is not just a threat to pyrethroid-only products but could also undermine the performance of newer classes of ITNs.

In addition to their importance in ITNs, pyrethroids were also historically widely used in IRS but since the publication of the Global Plan for Insecticide Resistance Management (WHO 2012), which strongly discouraged the use of the same active ingredients in ITNs and IRS, spray programmes have largely switched to organophosphates or carbamates (Tangena et al. 2020), and more recently neonicotinoids (Ngufor et al. 2017). Pyrethroids are also found in spatial repellents such as aerosols and coils (Bibbs et al. 2017) and are still widely used in agricultural applications; this intense use in endemic settings has inevitably led to the selection of

resistance (Churcher et al. 2016). The levels and geographical distribution of resistance in *Anopheles* mosquitoes has steadily increased since the beginning of the century, as reviewed in (Moyes et al. 2020) and there are increasing concerns about the impact this has on the efficacy of ITNs.

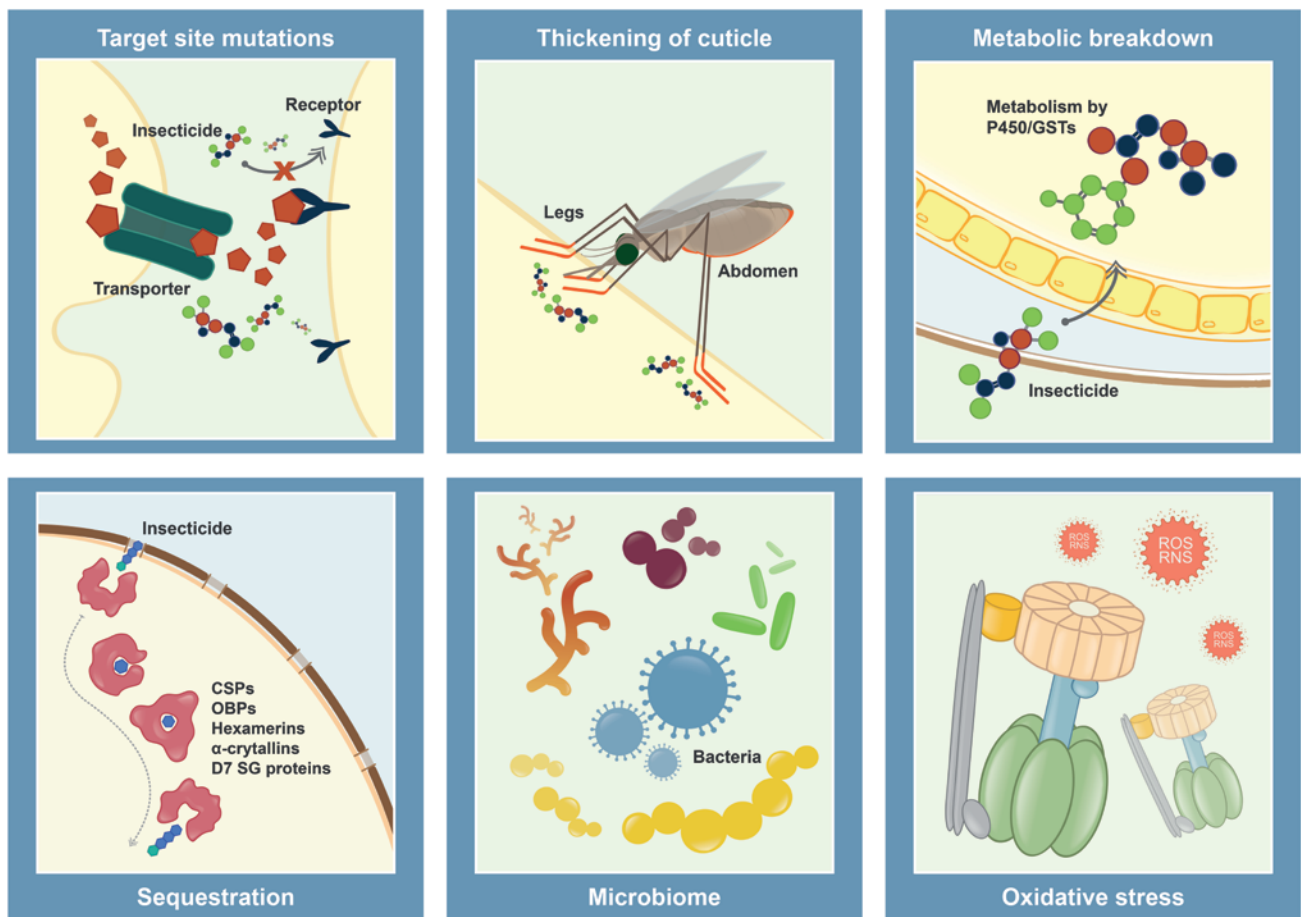
Understanding the mechanisms of pyrethroid resistance is essential for predicting patterns of cross resistance and making evidence-based decisions in insecticide resistance management, particularly in the context of dual active ingredient ITNs. Insecticides used in ITNs or IRS products must penetrate through the insects cuticle and travel through the body to reach and bind to their target, which for the pyrethroid class is a component of the nervous system, the voltage gated sodium channel (VGSC). Until recently, studies on the molecular basis of resistance focused primarily on two major mechanisms: mutations in the target site reducing insecticide binding, and increased rates of detoxication of insecticides by elevated activity of metabolic enzymes. The advent of lower cost, higher throughput genomic and transcriptomic sequencing, has unveiled additional mecha-

nisms (Fig. 1), the distribution and significance of which are the subject of investigation (Bayili et al. 2017; Staedke et al. 2020; Tiono et al. 2018). This review provides an update on current knowledge on the mechanisms conferring pyrethroid resistance in *Anopheles* mosquitoes, focusing primarily on the major African malaria vector, *Anopheles gambiae* (a species complex in which the most important vectors are *An. gambiae s.s.*, *An. coluzzii* and *An. arabiensis*); the review focuses on some of the more recently validated mechanisms and other putative mechanisms implicated by comparative gene expression studies. Current understanding of the regulatory processes controlling expression of insecticide resistance genes and pathways are briefly discussed.

## 2 Insecticide resistance mechanisms

### 2.1 Reduced penetrance-cuticular resistance

The cuticle is the first tissue insecticides need to efficiently penetrate to reach their target. The cuticle is structurally



**Fig. 1. Summary of insecticide resistance mechanisms in *Anopheles gambiae*.** Left to right, top row: Target site mutations are single nucleotide polymorphisms in the target site of the insecticide, reducing binding affinity; Thickening of the cuticle results in reduced penetrance of insecticide upon tarsal contact; Metabolic breakdown of insecticide results from increased levels of detoxification enzymes, such as cytochrome P450s and glutathione-S-transferases. Left to right, bottom row: Sequestration involves the binding of insecticides to reduce the amount reaching the target site; Differences in the microbiome have been identified in insecticide susceptible and resistant mosquitoes, but a causal link has yet to be shown; Changes in the redox state have been observed post pyrethroid exposure, and activation of oxidative stress sensing pathways, such as MafS-cnc may contribute to the resistance phenotype.

divided into different layers: the epicuticle, that is the outermost part and is mainly composed of hydrocarbons and other waxes, and the procuticle which accounts for most of the cuticular mass and is rich in chitin and proteins. Changes in the thickness or composition of each of these layers have been described in different insect species and linked to resistance, by lowering the insecticide penetration rate (reviewed in Balabanidou et al. 2018). In *An. gambiae* a thicker epicuticle, containing 30% more hydrocarbons, was associated with a 50% reduction in the penetration rate of radiolabelled deltamethrin after exposure to insecticide impregnated WHO papers (Balabanidou et al. 2016). In another resistant *An. gambiae* population, thickening of all cuticular layers was observed and associated with reduced deltamethrin penetration, after topical application of the insecticide on the mosquitoes' legs (Yahouédo et al. 2017). Over-expression of cuticular proteins with chitin binding motifs has also been reported in several resistant *Anopheles* populations and could be linked to resistance by increasing the pro-cuticles thickness or changing its physicochemical properties. This has been shown in *Cx. pipiens pallens*, where reduction in expression of CPLCG5 by RNA interference resulted in a thinner cuticle with unorganized laminae, as observed through transmission electron microscopy, and increased knock-down after pyrethroid exposure (Huang et al. 2018). As the insect cuticle plays a critical role in the physiology of insects, changes in its composition or thickness could affect multiple traits. For example, cuticular hydrocarbons have been associated with protection from desiccation and pathogens, and act as pheromones in several insects (Howard & Blomquist 2005). Thus, increased levels of hydrocarbons could affect different aspects of the mosquitoes' behaviour and physiology and impact their fitness (Chung & Carroll 2015; Adams et al. 2021).

Although the cuticular hydrocarbon (CHC) biosynthetic pathway has been described in *An. gambiae* and candidate genes have been proposed for each step (Grigoraki et al. 2020) (a subset of which has also been functionally validated to play a role in CHC biosynthesis (Balabanidou et al. 2016; Grigoraki et al. 2006)), the mechanisms underlying increased production or deposition of CHC on the cuticle remain largely unknown. Likewise, a causative link between the over-expression of specific cuticular proteins and cuticular resistance has not yet been established. This has so far impeded the identification of genetic markers for cuticular resistance that could be used in molecular diagnostics to screen field populations. The absence of genetic markers in combination with the technically advanced methods required to identify cuticular modifications (Scanning and Transmission Electron Microscopy and GC-MS analysis) and the inherent plasticity of the cuticle, that is affected by multiple environmental, physiological and genetic factors (Caputo et al. 2005; Cheng et al. 2018; Polerstock et al. 2002), makes the characterisation of this resistance mechanism challenging.

## 2.2 Mutations on the insecticides target site

Pyrethroids target the voltage-gated sodium channel (VGSC), a key component of the insects' nervous system. The VGSC consists of four homologous domains (DI-DIV) each of which has six transmembrane segments (S1–S6). When pyrethroids bind to the channel they stabilize its ion-conducting active state, thus preventing its inactivation (Bloomquist 1996). This results in the disruption of the nerve cells normal function and causes paralysis and eventually death (Bloomquist 1996). Mutations at the VGSC that reduce the binding affinity of pyrethroids confer resistance to this knock-down effect. The first mutations described in pyrethroid resistant *An. gambiae* were two single-base-pair substitutions at codon 995 (widely known as L1014F and L1014S, based on the *Musca domestica* numbering) within segment 6 of domain II, resulting in substitution of leucine with either phenylalanine or serine (Martinez-Torres et al. 1998). These mutations are widespread in field populations and their frequency has reached high levels, even fixation in some areas (Jones et al. 2012). Molecular diagnostics have been developed for these classical knock-down resistance (kdr) mutations and are routinely used in insecticide resistance monitoring programs to predict the presence of pyrethroid and DDT resistance in field populations (Bass et al. 2007). More recently the effect size of the L995F mutation was evaluated *in vivo* by introducing it through CRISPR in a fully susceptible *An. gambiae* genetic background (Grigoraki et al. 2021). When in homozygosity, the mutation conferred 9-to-20-fold resistance to pyrethroids and > 25 fold resistance to DDT (Grigoraki et al. 2021). Fitness disadvantages were also observed in the genome modified line, including increased mortality at the larval stage and a reduction in fecundity and adult female longevity (Grigoraki et al. 2021). Thus, in the absence of additional mechanisms, that can compensate for these fitness costs, a reduction in the frequency of this mutation is expected if the selection pressure from insecticides is withdrawn.

Analysis of whole genome sequencing data have revealed a number of additional substitutions on the VGSC, several of which have since been shown to be linked to resistance (Clarkson et al. 2021). Some of these mutations occur almost exclusively on haplotypes carrying the known L995F kdr allele. Thus, these mutations could provide a further selective advantage, by either enhancing the protective effect of L995F, as has been shown for mutation N1570Y (Jones et al. 2012), or compensating for its fitness costs. Other substitutions including the paired I1527T and V402L (I1532T and V410L, based on the *M. domestica* numbering) substitutions, which show signs of positive selection, have not been found on the same haplotype as the classical L995F and likely confer knock down resistance on their own. Indeed, mutation V402L was functionally validated by generating a CRISPR/Cas9 genome modified *An. gambiae* strain carrying this mutation in a fully insecticide susceptible genetic background (Williams et al. 2022a). The

mutation was shown to confer 2-to-7-fold resistance to pyrethroids and 4-fold resistance to DDT (Williams et al. 2022a). This mutation has been identified in other arthropod species (Dong et al. 2014; Haddi et al. 2017) and has been shown, in the *Xenopus* oocyte system, to reduce sensitivity of sodium channels to type I and II pyrethroids (Dong et al. 2014). This highlights that absence of mutations at position L995 do not necessarily mean absence of target site resistance in a population.

The use of CRISPR to generate genome modified lines that carry specific *vgsc* mutations on a defined genetic background gives us the unique opportunity to experimentally evaluate and compare their effect on insecticide resistance and other traits that determine mosquitoes' fitness. This methodological advancement enables us to study the VGSCs genetic variation, that is more complex than originally thought (Clarkson et al. 2021).

### 2.3 Detoxification genes

Comparative transcriptomic analysis of insecticide resistant and susceptible *An. gambiae* mosquitoes, either through quantitative PCR, microarray or Next Generation Sequencing, repeatedly shows the over-expression of detoxification genes in resistant mosquitoes. Among these, cytochrome P450s are most commonly associated with pyrethroid resistance. P450s mediate resistance by hydroxylating the insecticide molecules, making them less toxic and easily excretable (Xu et al. 2005).

Several *An. gambiae* P450s (recently reviewed in Vontas et al. 2020), including CYP6M2, CYP6P3, CYP9K1 and CYP6P4 have been expressed as functional recombinant enzymes in bacteria or insect cell lines and their ability to metabolise insecticides has been validated *in vitro*. Kinetic analysis has also been performed in some cases to evaluate their catalytic efficiency, but as pointed out by Vontas et al. 2020 comparing values between P450s and making inferences on their relative effect *in vivo* needs to be done with caution. The contribution of a detoxification enzyme in resistance is likely determined by multiple factors including its catalytic efficiency, but also its tissue localization and levels of expression.

Another important characteristic of P450s in relation to insecticide resistance management is their plasticity. Some enzymes are able to metabolise insecticides from different insecticide classes. For example, *in vitro* assays have shown that CYP6M2 and CYP6P3 are able to metabolise pyrethroids, but also organophosphates and pyriproxyfen, an insect growth regulator (Yunta et al. 2016, 2019). Detoxification enzymes active against a broad range of insecticides can cause cross-resistance, and thus reduce the efficiency of resistance management strategies that are based on applying insecticides from different classes in rotation.

The role of the *An. gambiae* P450s, CYP6M2 and CYP6P3 in insecticide resistance has been functionally validated *in vivo*. Ubiquitous over-expression of these two

P450s, through the Gal4-UAS system, either in the model organism *Drosophila melanogaster* or directly in *An. gambiae* confers reduced susceptibility to pyrethroids (Adolfi et al. 2019; Edi et al. 2014). Accruing evidence on the importance of P450s in insecticide resistance has fuelled the search for compounds that can inhibit their function. Such compounds could be used as synergists to, at least partially, alleviate the problem of resistance. Indeed piperonyl butoxide (PBO), a chemical that inhibits mixed-function oxidases, has been incorporated in ITNs, which have shown increased efficacy (compared to pyrethroid-only treated nets) in areas with high levels of resistance (Gleave et al. 2017).

Elevated expression of genes from other detoxification gene families have also been associated with pyrethroid resistance, including esterases, glutathione transferases and ABC-transporters but these are generally found in conjunction with elevated P450 activity; these gene families may be important in the detoxification and/or transportation of pyrethroid metabolites.

### 2.4 Sequestration

Insecticide sequestration is another mechanism by which the amount of insecticide reaching and binding to its target site, can be reduced. Recent work has shown that this resistance mechanism may be important and mediated by multiple protein families, including chemosensory proteins, odorant binding proteins, D7 salivary gland proteins, and hexamerins (Ingham et al. 2018).

#### 2.4.1 Chemosensory proteins

Chemosensory proteins include odorant binding proteins (OBPs) and chemosensory proteins (CSPs). Both families have a diverse range of functions, including responding to external chemical stimuli (reviewed in Pelosi et al. 2017). Recently, a CSP, SAP2, has been shown to be a key gene involved in pyrethroid resistance in *An. gambiae* and *An. coluzzii* mosquitoes in West Africa (Ingham et al. 2019). SAP2 is up-regulated at transcript level both constitutively and post-pyrethroid exposure, binds with high affinity to pyrethroids, and perturbation of expression through up-regulation or knockdown has significant impacts on resistance. Further, SAP2 is enriched in tissues that directly contact insecticide-treated surfaces such as legs and mouth parts. Up-regulation of CSPs has been detected in multiple *Anopheles coluzzii* populations from the Sudan-Sahel region (Ibrahim et al. 2022) and direct links with resistance through transgenics (Li et al. 2021; Xu et al. 2022) and binding assays (Li et al. 2017; Lin et al. 2018; Xu et al. 2022) have been shown across multiple arthropod species.

In addition to CSPs, OBPs have been linked to pyrethroid resistance in *An. gambiae* through transcript overexpression across multiple resistant populations (Bonizzoni et al. 2015; Ibrahim et al. 2022; Ingham et al. 2018; Kefi et al. 2021; Messenger et al. 2021; Williams et al. 2022b). Although no studies show a direct link between OBPs and resistance in

*Anopheles* mosquitoes, knockdown of OBP28 in the mosquito *Cx. quinquefasciatus* leads to increased susceptibility to the pyrethroid deltamethrin (Shen et al. 2022), whilst binding studies have shown interactions of these proteins with insecticides (Liu et al. 2020; Zhang, Lan et al. 2020; Zhang, Xu et al. 2020; Zhang et al. 2021), indicating that this family warrants further study in *Anopheles*.

#### 2.4.2 D7 salivary gland proteins

Overexpression of two D7 salivary gland proteins (SGPs, D7r4 and D7r2) was first noted in bendiocarb resistant mosquitoes from Uganda and molecular docking demonstrated putative binding of this insecticide to D7r4 (Isaacs et al. 2018). Subsequent studies have linked D7 SGPs with pyrethroid resistance in *An. funestus*, specifically D7r4 and D7r3, whilst demonstrating down regulation of these transcripts in sympatric *An. coluzzii* (Elanga-Ndille et al. 2019). Mining of transcriptomic data demonstrates that these genes are often found up-regulated in resistant compared to susceptible *Anophelinae* (Ingham et al. 2018; Isaacs et al. 2018) and recent data shows D7r4 as one of the most highly expressed transcripts in both malathion and pyrethroid resistant *An. arabiensis* from Ethiopia (Messenger et al. 2021). Interestingly, these proteins have secondary and tertiary structures showing similarities to OBPs in *Anophelinae* (Calvo et al. 2002), implying a putative role in sequestration. Perhaps surprisingly given the nomenclature, a recent study has shown increased expression of D7r2 in the legs of resistant mosquitoes compared to susceptible populations (Kefi et al. 2021) hinting at a role outside of the salivary gland.

#### 2.4.3 Hexamerins and $\alpha$ -crystallins

Hexamerins are one of the most abundant proteins in larval haemolymph and were originally identified as storage proteins that typically disappear after eclosion; however, some members of the hexamerin family play important roles in adult insects (Martins & Bitondi 2016; Xuguo et al. 2006). Alpha-crystallins are members of the small heat-shock protein family being activated in response to stress and acting as chaperones to protect correct protein folds (Basha et al. 2012). Members of the hexamerin and alpha-crystallin family are up-regulated in some pyrethroid resistant *An. coluzzii* (Ibrahim et al. 2022; Ingham et al. 2018; Williams et al. 2022b) and perturbation of their expression through RNAi resulted in increased mortality post-exposure (Ingham et al. 2018). A recent RNAseq study on five resistant colonies from Burkina Faso demonstrated up-regulation of these families across multiple species, with the upregulation of 7 hexamerins being particularly striking in a resistant *An. arabiensis* population (Williams et al. 2022b), which was also seen in resistant *An. arabiensis* populations in Ethiopia (Messenger et al. 2021). Further, hexamerins have previously been linked to bti resistance in *Cx. quinquefasciatus* mosquitoes (Poopathi et al. 2014) where they are hypoth-

esised to form aggregates around the toxin (Ma et al. 2005), whilst heat shock proteins have been shown to have a role in resistance in agricultural pests (Dong et al. 2021; Li et al. 2017; Lu et al. 2017).

As with the microarray era, the decrease in cost of RNAseq experiments are resulting in an invaluable bank of data which needs to be explored in a holistic fashion. However, whilst transcriptomic studies are valuable for identifying novel genes or gene families associated with resistance, the long lists of differentially expressed transcripts remain a hurdle for functional validation. Through literature review it is clear that there are a number of commonalities across datasets, with several transcripts consistently identified as being amongst the most overexpressed transcripts in resistant *Anopheles* mosquitoes, but, as yet, with no published studies reporting their potential contribution to the phenotype. Amongst these are genes in the trypsin/chymotrypsin (Abdalla et al. 2014; Ibrahim et al. 2022; Kwiatkowska et al. 2013; Toé et al. 2015; Wilding et al. 2015; Wipf et al. 2022; Wondji et al. 2022), aquaporin (Ibrahim et al. 2022; Toé et al. 2015) and the UGT families (Antonio-Nkondjio et al. 2016; Ibrahim et al. 2022; Kouamo et al. 2021; Nkya et al. 2014; Tene et al. 2013; Williams et al. 2022b; Wipf et al. 2022; Wondji et al. 2022).

### 3 Other putative resistance mechanisms

#### 3.1 Microbiome

Resistance is largely considered in the context of genetic traits of the vector; however, the microbial composition of both the internal and the surface microbiome have recently been linked with the pyrethroid resistance status of *Anopheles* mosquitoes (Dada et al. 2018, 2019; Ingham, Tennessen, et al. 2021; Omoke et al. 2021; Pelloquin et al. 2021). 16S profiling of knocked down and resistant *An. coluzzii* from Côte d'Ivoire revealed distinct differences in microbial composition, with *Serratia* and *Asaia* significantly overabundant in mosquitoes knocked down after exposure to deltamethrin, whilst *Ochrobactrum*, *Lysinibacillus* and *Enterobacteriaceae* are associated with pyrethroid resistance (Pelloquin et al. 2021). Similarly, whole genome sequencing found *Serratia* over-represented in pyrethroid susceptible mosquitoes, whilst *Elizabethkingia* is associated with resistance (Ingham, Tennessen, et al. 2021). Neither *Ochrobactrum* nor *Asaia* showed significant associations with resistance or susceptibility in this study (Ingham, Tennessen, et al. 2021). A similar study on *An. gambiae* demonstrated *Sphingobacterium*, *Lysinibacillus* and *Streptococcus* are associated with resistant mosquitoes in Kenya (Omoke et al. 2021). Studies on the new world malaria vector *An. albimanus* also revealed bacteria significantly associated with the pyrethroid resistance phenotype (Dada et al. 2018, 2019). Indeed, several of the genera associated with mosquitoes have been shown to be capable of

directly metabolising insecticides (Cycóń, Źmijowska and Piotrowska-Seget 2014; Guo et al. 2021; Jin et al. 2014; Zhan et al. 2018; Zhang, Wang, and Yan 2011) and colonisation of pest midguts by these bacteria have been shown to directly confer resistance (Cheng et al. 2017; Ishigami et al. 2022; Yoshitomo et al. 2012). In addition to direct impacts on the resistance phenotype, work in *An. arabiensis* (Barnard et al. 2019) and *An. stephensi* (Soltani et al. 2017) has linked antibiotic treatment with increased mortality post-exposure, through activity of endogenous detoxification enzymes, indicating microbiota play a role in inducing signalling. Although there is evidence for the role of the microbiome in resistance, the precise contributions of individual bacterial species remain elusive, due to complexity in deciphering the environmental variations in the microbiome from those with a directed contribution to resistance.

### 3.2 Oxidative stress

Oxidative stress has been linked with resistance and longevity both phenotypically and through transcriptional changes (Champion and Xu 2018; Müller et al. 2008; Oliver and Brooke 2014). Artificial manipulation of the redox state through feeding of pro-oxidants demonstrated a clear increase in susceptibility to DDT and permethrin as well as reduced fecundity (Champion and Xu 2018). Furthermore, increases in respiration have been demonstrated in pyrethroid resistant compared to susceptible mosquitoes, whilst exposure to pyrethroid insecticide causes a drop in this rate (Ingham, Tennessen, et al. 2021), phenotypes previously reported in agricultural pests (Guedes et al. 2006; Vinha et al. 2021). Underlying transcriptomic data supports the phenotypic changes through decrease in expression of respiration-related transcripts post-exposure, specifically those of the oxidative phosphorylation pathway (Ingham, Tennessen, et al. 2021; Ingham, Brown, and Ranson 2021). These data indicate that oxidative stress plays a role in, or is a result of, resistance to pyrethroids. The underlying decrease of the oxidative phosphorylation pathway and the decrease in respiration post-exposure hints at mosquitoes displaying metabolic plasticity in response to external stress.

## 4 Expression of resistance-related transcripts

### 4.1 Constitutive transcriptional response

Genetic control of transcription of resistance-related transcripts is poorly defined in *Anopheles* mosquitoes. A number of QTL studies have suggested the presence of both *cis* and *trans*-acting factors associated with pyrethroid resistance in *Anopheles* (Ranson et al. 2004; Witzig et al. 2013; Wondji et al. 2007) but delineation of the key genetic changes within these QTL has met with mixed success; in *An. funestus*, QTL mapping coupled with *in vitro* promoter characterisa-

tion have identified the *cis*-regulatory region for the major pyrethroid metabolisers CYP6P9a (Wondji et al. 2007) and CYP6P9b (Mugenzi et al. 2019), enabling the identification of DNA based markers to detect this metabolic resistance mechanism. However, the equivalent mutations in the regulatory regions of the key resistance associated P450s in *An. gambiae* remain elusive.

Similarly, genome wide association studies to identify resistance associated markers have proved challenging. Extremely high levels of polymorphism, extensive population substructuring and low levels of linkage disequilibrium pose methodological challenges for this approach (Weetman et al. 2018a) and, whilst some markers have been identified, their geographical reach appears limited.

Recently, transcriptional regulation of pyrethroid resistance by miRNAs has been suggested (Xu et al. 2022) but these also remain poorly characterised and epigenetic regulation of resistance in Anophelines is currently unstudied.

### 4.1.1 Copy number variations

Copy number variations (CNV) have been proposed as a major adaptive polymorphism contributing to insecticide resistance in multiple insect species (Weetman et al. 2018b). Many copy number variations are amplifications or duplications across known insecticide-resistance associated genes, such as cytochrome P450s and GSTs. Over 250 genes containing CNVs have been identified in the *Anopheles* genome using sequencing data from the 1000 genomes project (Lucas et al. 2019). The authors determined that CNVs were enriched for genes involved in metabolic detoxification and demonstrated these regions were under positive selection (Lucas et al. 2019). A similar study focused on organophosphate resistance and demonstrated a link between this phenotype and a CNV across the *Ace-1* locus (Grau-Bové et al. 2021). CNVs have also been linked with insecticide resistance in *An. funestus* through duplication of the CYP6P9 locus, the major causative gene associated with pyrethroid resistance in this species (Weedall et al. 2020).

### 4.2 Induced transcriptional response

Inducible expression of detoxification activity following xenobiotic exposure is a well-known phenomenon and transcriptomic data has demonstrated that sublethal insecticide exposure in *Anopheles* results in wide-ranging changes to underlying gene expression (Ingham, Brown and Ranson. 2021; Kefi et al. 2021; De Marco et al. 2017; Vontas et al. 2005) and that specific families known to be involved in direct binding or detoxification of insecticides are induced by exposure (Epis et al. 2014; Ingham et al. 2019; Kouamo et al. 2021; Mastrantonio et al. 2019). Taken together, these data indicate that in addition to constitutive overexpression of transcripts involved in insecticide resistance, signalling pathways further induce their expression.

#### 4.2.1 Transcription factors

Transcriptional control of exogenous stress is well described in insects (Gao et al. 2022). The cnc-MafS (also known as cnc-Nrf2) pathway, which responds to cellular oxidative stress and induces transcription of genes controlled by a xenobiotic response element, is one of the best studied and is up-regulated in multiple *An. gambiae* resistant populations (Ingham et al. 2018; Wipf et al. 2022). Perturbation of MafS significantly increases susceptibility to pyrethroid insecticides and DDT whilst increasing resistance to malathion, a phenomenon termed negative cross resistance (Ingham et al. 2017). Transcriptomics on MafS knockdown revealed that this pathway-controls expression of a number of known resistance-related transcripts, including the pyrethroid metaboliser CYP6M2 which is also thought to convert the pro-insecticide malathion to its active form, putatively indicating a mechanism for negative cross-resistance (Ingham et al. 2017). The cnc- MafS pathway is also known to regulate resistance through control of expression of cytochrome p450s and GSTs in a number of other insect species, reviewed here (Wilding 2018). Further, a selective sweep around Keap1, the regulator of this pathway, is apparent within the *Anopheles* 1000 genomes project (Consortium 2017), indicating an importance in wild caught mosquitoes.

A recent study attempted to describe the network of transcription factors involved in insecticide response and described an additional 23 potential transcription factors involved in response to pyrethroid exposure (Ingham, Elg, et al. 2021).

#### 4.2.2 G-protein coupled receptors (GPCRs)

GPCRs play important roles in cell biology and signalling, and their function and potential utility as a direct target for insecticides has just been reviewed in (Liu et al. 2021). The first concrete link between GPCRs and pyrethroid resistance was described in *Cx. quinquefasciatus* through use of RNAi, when the knockdown of four GPCRs was shown to directly impact pyrethroid resistance (Li et al. 2014). It was suggested that the reduction in resistance was due to down-regulation of cytochrome P450s, which was confirmed in subsequent studies expanding the repertoire of GPCRs involved in resistance to include rhodopsin-like GPCRs and describing a putative signalling pathway (Li et al. 2015; Li and Liu 2017). GPCRs have thus far been overlooked in insecticide resistance in *Anopheles* mosquitoes; however, up-regulation of GPCR expression has been seen both in legs and whole organisms after deltamethrin exposure (Ingham. Brown and Ranson. 2021; Kefi et al. 2021).

## 5 Conclusion

Multiple mechanisms can confer pyrethroid resistance in malaria vectors. Given the recent increase in selection pressure imposed by the scale up in ITN use, many of these mechanisms may be relatively recent and hence may have

high fitness costs. Evolutionary genomics will likely increase our understanding of the origin, rate of spread and selective advantage conferred by each mechanism, but functional studies are also important to validate resistance associated markers and identify any synergism or antagonism between mechanisms. Similarly, bioassays will remain important in determining how combinations of these mechanisms may impact the performance of pyrethroids and other classes of insecticides both applied singly, and in combination as found in dual active ingredient products just now entering the market. Furthermore, not mentioned in this review due to lack of molecular insights, but of critical importance, are changes in vector behaviour linked to insecticide resistance which must be understood to maximise intervention impact.

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