Evaluating the Evidence from Molecular Structure and Population Studies for Cross-Resistance to the Pyrethroids Used in Malaria Vector Control

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Abstract

The primary malaria control intervention in high burden countries is the deployment of long-lasting insecticide-treated nets (LLINs) treated with pyrethroids, alone or in combination with a second active ingredient or synergist. It is essential to understand whether the impact of pyrethroid resistance can be mitigated by switching between different pyrethroids or whether cross-resistance precludes this. Structural diversity within the pyrethroids could mean some compounds are better able to counteract the resistance mechanisms that have evolved in malaria vectors. Here we consider variation in vulnerability to the P450 enzymes that confer metabolic pyrethroid resistance in *Anopheles gambiae* s.l. and *Anopheles funestus*. We assess the relationships among pyrethroids in terms of their binding affinity to key P450s and the percent depletion by these P450s, in order to identify which pyrethroids diverge from the others. We then investigate whether these same pyrethroids also diverge from the others in terms of resistance in vector populations. We found that etofenprox, which lacks the common structural moiety of other pyrethroids, potentially diverges from the commonly deployed pyrethroids in terms of P450 binding affinity and resistance in malaria vector populations, but not depletion by the P450s tested. These results are supplemented by an analysis of resistance to the same pyrethroids in *Aedes aegypti* populations, which also found etofenprox diverges from the other pyrethroids in terms of resistance in wild populations. In addition, we found that bifenthrin, which also lacks the common structural moiety of most pyrethroids, diverges from the commonly deployed pyrethroids in terms of P450 binding affinity and depletion by P450s. However, resistance to bifenthrin in vector populations is largely untested. The prevalence of resistance to the pyrethroids α-cypermethrin, cyfluthrin, deltamethrin, λ-cyhalothrin, and permethrin was correlated across malaria vector populations and switching between these compounds as a tool to mitigate against pyrethroid resistance is not advised without strong evidence supporting a true difference in resistance.

Introduction

The primary malaria control intervention in high burden countries is the deployment of long-lasting insecticide-treated nets (LLINs) treated with pyrethroids, alone or in combination with a second active ingredient or synergist [1, 2]. Widespread and increasing resistance to pyrethroids is, therefore, a serious potential threat to malaria control [3, 4]. Because the options for LLINs are limited, it is essential to understand whether the impact of resistance can be mitigated by switching between different pyrethroids or whether cross-resistance precludes switching. Pyrethroids listed by the World Health Organization (WHO) for malaria control are differentiated into two groups based on biological activity that is associated with the absence (Type I) or presence (Type II) of an alpha-cyano group (Fig. 1). Type II pyrethroids are more lethal to insects because of their higher potency to the voltage-gated sodium channel (VGSC) in nerve membranes, the primary target site of pyrethroids [5, 6]. The higher potency of Type II pyrethroids such as deltamethrin and α-cypermethrin translates into much lower doses being required to treat vector control products compared with Type I pyrethroids such as permethrin. This has led to increased deployment of alpha-cyano pyrethroids, in particular α-cypermethrin, which is currently
used in 28% of the prequalified vector control products [2]. Generally, pyrethroids used in vector control possess the common structural motif of phenoxy benzyl alcohol coupled with a cyclopropane ring via an ester bond, except for bifenthrin and etofenprox (Fig. 1). This narrow spectrum of chemical variation among pyrethroids makes it likely that cross-resistance will occur in malaria vector populations.

The high burden countries where LLINs are deployed are concentrated in Africa where the most important vectors are *Anopheles gambiae* s.l. and *Anopheles funestus* [7]. Pyrethroid resistance in malaria vectors is primarily associated with target-site insensitivity due to mutations in the *Vgsc* gene known as knockdown resistance (*kdr*) and increased detoxification activity known as metabolic resistance. Metabolic mechanisms of resistance are found in all African malaria vectors whereas *kdr* mutations are common in species of the *Anopheles gambiae* complex but not in the *An. funestus* subgroup [8–14]. There are multiple amino acid substitutions that cause target-site insensitivity resulting in pyrethroid resistance [15]. This includes a mutation, M918T, that produces a super-knockdown (*s-kdr*) phenotype in houseflies. Structure modelling studies in M918T phenotypes indicate that the highest degree of resistance in *s-kdr* houseflies depends on the chemical structure of the insecticide which is positively correlated with the presence of an α-cyano group coupled with a phenoxybenzyl moiety in the larger Type II pyrethroid molecules such as deltamethrin and fenvalerate [16]. By comparison, the most common *Vgsc* resistance allele in west African *An. gambiae* populations, L1014F, is not influenced by pyrethroid chemical structure when expressed alone in house flies [17].

Although *kdr* mutations are common in *An. gambiae* s.l., they may have a relatively modest impact on resistance, and they are absent from highly pyrethroid-resistant *An. funestus* populations, suggesting that metabolic mechanisms have a greater impact in African malaria vectors [18–20]. Metabolic resistance is most commonly mediated by elevated levels of cytochrome P450 enzymes [21]. Transcriptome-wide studies of gene expression in resistant and susceptible mosquito strains have found upregulation of several P450 genes is associated with resistance to both a Type I pyrethroid (permethrin) and a Type II pyrethroid (deltamethrin). For example, upregulation of the *CYP6P3* gene and its orthologues *CYP6P9a* and *CYP6P9b*, and of the *CYP6AA1*, *CYP6Z1* and *CYP6Z3* genes, is associated with resistance to both pyrethroids in *An. gambiae* / *An. coluzzii* and *An. funestus* [22–32]. In addition, upregulation of the *CYP6Z2* gene in *An. gambiae* and *An. coluzzii*, and the *CYP6M7* gene in *An. funestus*, is also associated with resistance to both pyrethroids [23–25, 27, 28, 30–32]. These findings from studies of gene expression in resistant and susceptible strains provide evidence for P450-mediated cross-resistance in Anopheles populations, particularly to deltamethrin and permethrin, but associations with resistance to more than one pyrethroid have not always been found, a limited range of pyrethroids have been tested, and these studies don’t give an indication of whether cross-resistance is stronger between some pyrethroids than others. Like the *Anopheles* vectors, target-site mutations and metabolic resistance are also thought to be the main resistance mechanisms in *Aedes* mosquitoes [33, 34].

An assessment of the impact of individual structural variation within the pyrethroid class on resistance in the field is required to inform the best use of different compounds. A previous study assessed resistance in malaria vector populations at over one thousand African sites and showed that when spatio-temporal
trends were separated from noise in the susceptibility test data, strong associations among the resistance trends for three structurally similar pyrethroids (deltamethrin, \( \alpha \)-cyhalothrin and permethrin) were found [35]. The variance in the mean percent mortality values was 28 for the west Africa model and 23 for the east Africa model, reflecting the noisiness of the mortality data. This study also noted that the prevalence of resistance to permethrin was typically higher than that to deltamethrin, however, caution is needed when interpreting differences found using susceptibility test data because they may be due to real differences in the prevalence of resistance or differences in the calibration of the diagnostic dose or both. Diagnostic doses currently recommended for use were calculated by doubling the dose of a compound which kills 100% of a susceptible strain of a species, or doubling the LC_{99} in this strain [36, 37]. A robust recommendation should be based on data from multiple strains in different testing centres, but where this is not possible doses may not be well calibrated between compounds. It is clear that differences in resistance between individual pyrethroids cannot be generally assumed, but it remains unclear whether meaningful differences can occur, particularly when a wider range of pyrethroid chemistries is considered.

Here we take a new approach to assess variation in resistance among pyrethroids. We first assess differences in pyrethroid chemistry that influence inhibition of the key enzymes that confer metabolic resistance in African malaria vectors, and the rate of depletion of each pyrethroid by these enzymes [38]. Of the primary resistance genes, the P450 superfamily is most frequently associated with metabolic resistance to pyrethroids in malaria vectors. Therefore, we assessed the relative differences among six pyrethroids in terms of their molecular interactions with P450 enzymes from the major African malaria vectors by constructing a P450s structure-activity relationships model (P450s-SAR). We focus on \( \alpha \)-cypermethrin, deltamethrin and permethrin as most relevant for recommendations regarding the current LLIN options. However, for broader future consideration, we include bifenthrin, etofenprox, cyfluthrin and \( \lambda \)-cyhalothrin, structurally varied pyrethroids that are also in the WHO’s prequalified list for malaria vector control (Fig. 1) [2]. We then analyse resistance to these pyrethroids in multiple vector populations to determine whether the relative differences found by P450s-SAR studies translate into relative differences in resistance within wild populations. This is supplemented by an analysis of resistance in arbovirus vector populations. Finally, the resistance associations found across insecticide classes are also analysed in order to put the relationships found within the pyrethroids into the wider context of cross-resistance generally and to further investigate whether cross-resistance predicted by laboratory studies can be detected as general trends in the field data.

**Material And Methods**

In order to test whether relationships identified by SAR studies can be detected in the field, we constructed dendrograms for the hierarchical relationships between pyrethroids found by a series of molecular and field studies, and then compared the dendrograms obtained.
Relationships among pyrethroids in terms of functional activity data

P450 inhibition assays using fluorogenic probe substrates have become commonplace in drug discovery screening cascades and are a rapid method of screening for insecticide interactions with mosquito P450s to predict insecticide binding, metabolism, cross-resistance and synergy [38–41]. In this study, the half maximal inhibitory concentration (IC$_{50}$), which provides a value for inhibition of each P450 by each pyrethroid, and the percent depletion, which gives a value for metabolism of each pyrethroid by each P450, were both included to establish a P450s structural activity relationship model. This model was used to understand the chemistry of the pyrethroids, and the interaction with mosquito P450s that function as monooxygenases in metabolic resistance and to predict cross-resistance liabilities in vivo. Low IC$_{50}$ values indicate the pyrethroid is a potent inhibitor that may be able to counter resistance mediated by P450s. Low percent depletion indicates low metabolism of the pyrethroid, which means it may be less vulnerable to resistance mediated by P450s.

The IC$_{50}$ values for permethrin, etofenprox and bifenthrin (Type I) and deltamethrin, λ-cyhalothrin and α-cypermethrin (Type II) pyrethroids that were exposed to recombinant P450s from the *An. gambiae* Kisumu strain (CYPs 6Z2, 6M2, 6P2, 6P3 and 9J5) and the *An. funestus* FUMOZ strain (CYP6P9a) were extracted from two studies [38, 41]. In addition, inhibition activity data for these pyrethroids exposed to CYP6Z3 from the *An. gambiae* Kisumu strain were also generated (Additional File 1).

The values for percentage depletion (metabolism) of each pyrethroid by three of the enzymes, CYP6M2, CYP6P3 and CYP6P9a, which were expressed in a single plasmid construct, were also extracted from the same sources and used for the comparative analysis.

The two datasets were analysed using hierarchical clustering of rows (insecticide) and columns (P450) by Perseus v1.6.14.0 to produce two visual heat maps representing the clustered matrices for relative insecticide binding affinity and insecticide vulnerability to metabolic attack. The clustered matrices for functional activity data for these six pyrethroids against these seven P450s were then used to construct dendrograms for the hierarchical relationships among the pyrethroids.

Relationships among pyrethroids in terms of susceptibility test mortality in malaria vector populations

We accessed a published database of insecticide resistance in African malaria vectors [14] and identified all instances where a mosquito sample from the field had been tested using two or more pyrethroids. Pairs of results were extracted, rather than instances where a sample had been divided between tests of three or more pyrethroids, because there were insufficient data from studies testing > 2 pyrethroids against a single mosquito collection. This provided 3,153 pairs of WHO susceptibility test results from samples of the *An. gambiae* complex. Only data that detected resistance to at least one pyrethroid were included. That is, results from samples that had 100% mortality to all pyrethroids tested were excluded.
We conducted a series of correlation analyses to assess how closely associated each pair of pyrethroids is in terms of resistance. The mean value for the Pearson’s correlation coefficient was calculated across 1,000 bootstrapped samples for each pyrethroid pair using SPSS Statistics v25. A Holm-Bonferroni correction was applied to identify significant correlations among the multiple tests conducted while avoiding false positives [42]. The mean correlation coefficients generated were ranked to identify the most and least closely correlated pyrethroids. These bootstrap mean correlation coefficients were used to construct a dendrogram of the hierarchical relationships among pyrethroids using the unweighted pair-group method using the arithmetic mean (UPGMA [43]), where the highest correlation coefficient indicated the most closely related pair.

The analyses conducted using data from An. gambiae s.l. samples were repeated using data from An. funestus subgroup, An. arabiensis, An. coluzzii, An. funestus and An. gambiae samples. The same approach was also used for susceptibility test data from Aedes albopictus and Ae. aegypti to investigate whether the same relationships could be detected in these vectors of arboviruses, as detailed in Additional File 3. There were much lower data volumes for the individual Anopheles species, compared to An. gambiae s.l., and a limited selection of pyrethroid pairs could be tested so no dendrograms were constructed from these data. Finally, the correlations between resistance to deltamethrin and resistance to insecticides from other classes were calculated in order to put the relationships found within the pyrethroids into the broader context of cross-resistance.

**Results**

**Relationships among pyrethroids in terms of functional activity data**

The six pyrethroids were categorised according to their inhibition of diethoxyfluorescein (DEF) metabolism by P450s as potent (IC$_{50}$ < 1 µM), moderate (IC$_{50}$ 1–10 µM) and weak inhibitors (IC$_{50}$ > 10 µM) [44]. Accordingly, all pyrethroids investigated show low to moderate binding to the P450 panel (Fig. 2A, Table S2). Bifenthrin had the lowest binding to the P450s panel examined (Fig. 2A, Table S2). The CYPs 6P3, 6M2 and 6P9a were selected for comparative metabolism analysis because they are commonly associated with pyrethroid resistance and amongst the earliest pyrethroid resistance markers to be functionally validated and most heavily used for in-vitro screening [29, 41, 45, 46]. All of the pyrethroids apart from bifenthrin were strongly metabolised by 6P3 and its orthologues 6P9a expressed from An. gambiae and An. funestus respectively (Fig. 2B, Table S3). However, lower metabolism profiles were observed with 6M2 expressed from An. gambiae (Fig. 2B, Table S3). Notably, etofenprox was strongly metabolised by 6P3, 6M2 and 6P9a. Overall, the metabolism data presented in Fig. 2B and Table S3 ranked etofenprox, deltamethrin and permethrin as the most vulnerable insecticides for metabolic attack by the three enzymes, followed by α-cypermethrin and β-cyhalothrin, and bifenthrin demonstrated the lowest vulnerability.
The dendrograms indicate that permethrin and deltamethrin are closely related whereas bifenthrin diverges from these pyrethroids, in terms of inhibition of P450s and metabolism by P450s (Fig. 2).

**Relationships among pyrethroids in terms of susceptibility test mortality in malaria vector populations**

Each of the 15 pairs of values for pyrethroid resistance within *An. gambiae* s.l. was significantly correlated (Table 1). That is, populations with a higher prevalence of resistance to one pyrethroid tended to have higher prevalence of resistance to the others (Figs. 3 and S2). The pyrethroid pairs were ranked from the most closely correlated pair, deltamethrin vs λ-cyhalothrin, to the most divergent pair, etofenprox vs λ-cyhalothrin (Table 1, Fig. 4A). The correlation coefficients were used to construct a dendrogram of the hierarchical relationships among these pyrethroids (Fig. 4B). Deltamethrin, λ-cyhalothrin, permethrin, cyfluthrin, and α-cypermethrin were closely related whereas etofenprox diverged from the other five pyrethroids.

| Rank | Pyrethroid pair              | N   | Mean r  |
|------|-----------------------------|-----|---------|
| 1    | deltamethrin vs λ-cyhalothrin | 597 | 0.774*  |
| 2    | permethrin vs cyfluthrin     | 62  | 0.752*  |
| 3    | permethrin vs λ-cyhalothrin  | 484 | 0.729*  |
| 4    | deltamethrin vs permethrin   | 1278| 0.726*  |
| 5    | α-cypermethrin vs cyfluthrin | 27  | 0.709*  |
| 6    | deltamethrin vs α-cypermethrin | 242 | 0.684*  |
| 7    | deltamethrin vs cyfluthrin   | 64  | 0.675*  |
| 8    | permethrin vs α-cypermethrin | 197 | 0.671*  |
| 9    | λ-cyhalothrin vs α-cypermethrin | 154 | 0.573*  |
| 10   | permethrin vs etofenprox     | 68  | 0.567*  |
| 11   | deltamethrin vs etofenprox   | 80  | 0.549*  |
| 12   | α-cypermethrin vs etofenprox | 42  | 0.507*  |
| 13   | etofenprox vs cyfluthrin     | 20  | 0.476*  |
| 14   | λ-cyhalothrin vs cyfluthrin  | 54  | 0.467*  |
| 15   | λ-cyhalothrin vs etofenprox  | 63  | 0.418*  |

N is the sample size and r is the Pearson's correlation coefficient. Significant results (at the 0.05 level with a Holm-Bonferroni correction) are denoted by *. Non-significant results are denoted n.s. The most closely
Comparison of pyrethroid relationships seen in molecular and field studies

The three dendrograms using i) resistance in field populations, ii) P450 inhibition and iii) depletion by P450s were re-constructed incorporating only the five pyrethroids that were included in all three analyses (Fig. 5). The dendrograms for P450 inhibition and vector population resistance both show that deltamethrin, λ-cyhalothrin, permethrin are most closely related to each other, then α-cypermethrin, and etofenprox is most divergent (Fig. 5A and 5B). The dendrogram constructed using values for insecticide depletion metabolism by 6P3, 6M2 and 6P9a reveals different relationships among these pyrethroids, although permethrin and deltamethrin are still closely related (Fig. 5C).

Correlations in pyrethroid resistance within malaria vector species

Across An. funestus subgroup communities, there were significant correlations between resistance to deltamethrin and λ-cyhalothrin, permethrin and λ-cyhalothrin, and deltamethrin and permethrin, and the same was true for the four species tested (Table 2, Figures S3 and S4). There were insufficient data to test the other pyrethroid combinations for the African malaria vector species. Across Ae. aegypti populations, resistance to cyuthrin, deltamethrin, λ-cyhalothrin and permethrin was significantly correlated whereas there were no significant correlations between these four pyrethroids and etofenprox (full results are given in Additional File 3).

In order to put the relationships found within the pyrethroids into the wider context of cross-resistance across the insecticide classes used for malaria vector control, the correlations between deltamethrin and six commonly used non-pyrethroid insecticides were also calculated. Significant correlations with the prevalence of resistance to DDT were found for species within the An. gambiae complex but not for An. funestus (Table S4 and Figure S5). No significant correlations were found between the prevalence of resistance to deltamethrin and that to bendiocarb or propoxur (carbamates), malathion, fenitrothion or pirimiphos-methyl (organophosphates) for species within the An. gambiae complex or An. funestus.
Table 2
Correlations between resistance to different pyrethroids in African malaria vector species.

| Deltamethrin vs λ-cyhalothrin | N   | r   |
|-------------------------------|-----|-----|
| Anopheles funestus subgroup   | 46  | 0.818* |
| Anopheles funestus            | 24  | 0.865* |
| Anopheles arabiensis         | 28  | 0.946* |
| Anopheles coluzzii           | 18  | 0.863* |
| Anopheles coluzzii/gambiae    | 19  | 0.603* |
| Anopheles gambiae            | 19  | 0.418 n.s. |

| Permethrin vs λ-cyhalothrin   |     |     |
|-------------------------------|-----|-----|
| Anopheles funestus subgroup   | 26  | 0.786* |
| Anopheles funestus            | 16  | 0.845* |
| Anopheles arabiensis         | 31  | 0.859* |
| Anopheles coluzzii           | 14  | 0.740* |
| Anopheles coluzzii/gambiae    | 17  | 0.790* |
| Anopheles gambiae            | 4   | Not tested |

| Deltamethrin vs permethrin    |     |     |
|-------------------------------|-----|-----|
| Anopheles funestus subgroup   | 113 | 0.608* |
| Anopheles funestus            | 69  | 0.726* |
| Anopheles arabiensis         | 116 | 0.840* |
| Anopheles coluzzii           | 48  | 0.793* |
| Anopheles coluzzii/gambiae    | 63  | 0.714* |
| Anopheles gambiae            | 75  | 0.782* |

Significant results (at the 0.05 level with a Holm-Bonferroni correction) are denoted by *. Non-significant results are denoted n.s. The term ‘Anopheles coluzzii/gambiae’ refers to mosquito samples that were undifferentiated between An. coluzzii (M form) and An. gambiae (S form), before they were recognised as two species.

**Variation in pyrethroid resistance within populations of African malaria vector species**
The results presented above show significant correlations in resistance among the pyrethroids tested, but this result does not preclude the possibility that the prevalence of resistance is generally higher in one pyrethroid compared to the others across populations with differing levels of pyrethroid resistance. The insecticide depletion data presented above indicates that some pyrethroids are potentially more vulnerable to P450 attack, particularly etofenprox which was most depleted by the three P450s (Table S3). This leads to the question of whether higher levels of resistance to this compound can be detected in wild mosquito populations. An analysis of the paired data from An. gambiae s.l. samples collected across Africa provides no evidence that the prevalence of resistance is consistently higher for etofenprox compared to the other pyrethroids in An. gambiae s.l. (Figure S6) but mortality was significantly lower after Ae. aegypti populations were exposed to etofenprox compared to mortality following exposure to deltamethrin, cyfluthrin, λ-cyhalothrin and permethrin (Table S7).

To put the mortality differences found among pyrethroids (Figure S6-S8) into the wider context of cross-resistance, the prevalence of resistance to deltamethrin was compared to the prevalence of resistance to six non-pyrethroid insecticides in paired susceptibility tests (Figure S9). A reversal in the differences between resistance to deltamethrin and to the organochlorine DDT was found, with An. gambiae s.l. species having significantly higher resistance to DDT whereas An. funestus had significantly higher resistance to deltamethrin. In all species tested, mortality was lower following deltamethrin exposure compared to bendiocarb and propoxur (carbamates), malathion, fenitrothion and pirimiphos-methyl (organophosphates) exposure.

**Discussion**

The results of this study highlight which of the pyrethroids used in malaria control are closely related in terms of inhibition of and depletion by P450s. Other studies of structurally diverse pyrethroids have also shown variation in P450 metabolism of pyrethroids with different structure. An *in vivo* study of the An. funestus strain, FUMOZ-R, which is characterised by upregulated P450 levels without any target site mutations, found that transfluthrin, which contains a polyfluorobenzyl alcohol, was effective in the absence of the generic P450 inhibitor, piperonyl butoxide (PBO), whereas the other pyrethroids that contain common phenoxy benzyl moiety including cypermethrin, β-cyfluthrin, deltamethrin and permethrin were only effective when partnered with PBO [47]. This effect was associated with an inability of detoxifying enzymes to bind to the uncommon structure of transfluthrin. A similar observation was reported earlier from agriculture where an isogenic metabolic resistance strain isolated from a pyrethroid-resistant field population of *Helicoverpa armigera* showed significant cross-resistance between the pyrethroids characterised by having both the phenoxy benzyl and aromatic acid moieties whereas the substitution of the phenoxybenzyl group with a polyfluorobenzyl group, as occurs in tefluthrin, benfluthrin and transfluthrin, overcame most of this resistance [48]. These studies support the aim of identifying pyrethroids that are active against resistant populations when P450-mediated resistance plays a major role. In our study, bifenthrin diverged from the other pyrethroids in terms of both inhibition and depletion by P450s, but no susceptibility test data were available for resistance to bifenthrin in populations of African malaria vectors. Susceptibility test data were available for etofenprox and it was found to diverge
from the more commonly deployed pyrethroids in terms of inhibition of *An. gambiae* and *An. funestus* P450s, and in terms of resistance in *An. gambiae* s.l. and *Ae. aegypti* populations.

The susceptibility test data from these populations show strong associations between resistance to the most commonly used pyrethroids (deltamethrin, λ-cyhalothrin, permethrin and α-cypermethrin), in agreement with the results for binding affinity and with earlier studies of spatio-temporal trends in *An. gambiae* s.l. [3, 35]. The correlations in resistance among these pyrethroids, which were demonstrated in all the major African malaria vectors, suggest that if differences in resistance to these pyrethroids (as well as the less commonly deployed cyfluthrin) are found using susceptibility tests conducted on a small number of field samples of malaria vectors, further evidence should be obtained before any decision is made to switch between them.

Greater differentiation was found for resistance to bifenthrin in terms of both inhibition and depletion by P450s. The results for bifenthrin are interesting because they show that 1) this pyrethroid differs from the other pyrethroids in terms of P450 binding and metabolism, and 2) it may be less susceptible to common P450 enzymes. Bifenthrin is the active ingredient in one indoor residual spray (IRS), Bistar 10WP [2, 49], which is used in India. Bifenthrin IRS was trialled in Nigeria in 2006 and Zambia in 2011 [50–52] but has not been widely deployed in Africa where concerns about the duration of residual activity have been raised [52–54]. There are no field data from susceptibility tests on African malaria vectors conducted using bifenthrin, presumably because this compound is rarely deployed and because there is no recommended diagnostic dose for use in a susceptibility test. One study of *Anopheles sinensis* in Korea collected blood-fed adults in the field and exposed the F1 larvae to each of the pyrethroids considered by our study. They calculated resistance ratios using LC	extsubscript{50} values from a susceptible strain and found that the larvae were most susceptible to bifenthrin, cyfluthrin and etofenprox, in that order, and least susceptible to permethrin [55]. Further evidence comes from studies of *Aedes* vectors, including three studies that tested bifenthrin [34]. One study in Mexico tested seven populations of *Aedes aegypti* with eight pyrethroids and compared the concentrations required for 50% knockdown (KC	extsubscript{50}) and mortality (LC	extsubscript{50}) to the same values obtained using a susceptible strain to give a resistance ratio (RR) [56]. Across the seven populations, resistance to deltamethrin, lambda-cyhalothrin, permethrin and α-cypermethrin were highly correlated (in terms of both RRKC	extsubscript{50} and RRLC	extsubscript{50}), indicating the existence of strong cross-resistance. However, the resistance values for bifenthrin were not correlated with any those for the other four compounds and the study concluded bifenthrin could be an alternative insecticide for *Ae. aegypti* in Mexico. Two independent studies in Thailand tested three *Ae. aegypti* and three *Ae. albopictus* populations, respectively, and calculated the diagnostic doses for each pyrethroid including bifenthrin using a susceptible strain [57, 58]. In both instances, the population with the highest deltamethrin resistance also had the highest bifenthrin resistance, so no evidence for divergence in resistance was observed for these two species in Thailand. Given the known data noise in susceptibility test results, caution is needed when interpreting the results from a single study at a small number of sites. It is also worth noting that bifenthrin’s relative immunity to depletion by CYP6M2, CYP6P3 and CYP6P9a described here was not found when tested previously [28]. Metabolism assays conducted by two earlier studies
showed that CYP6M7, CYP6P9a and CYP6P9b from An. funestus metabolized bifenthrin (62%, 68% and 71% respectively) as well as permethrin, deltamethrin and λ-cyhalothrin (ranging from 46–81% depletion). Field tests for bifenthrin resistance in malaria vector populations are needed before we can reach a firm conclusion about whether bifenthrin should be recommended in situations where resistance to other pyrethroids has been found.

The analyses of binding affinity data and of field data from malaria vector populations both show that resistance to etofenprox diverges, to a degree, from resistance to the more commonly deployed pyrethroids. This result is backed up by data from studies of resistance in Ae. aegypti. However, the depletion activity data suggests that etofenprox is more vulnerable to P450 metabolism and if resistance to this compound is found to be greater in malaria vector populations then a switch would not be advised. A trend for higher resistance to etofenprox was not seen in the data from malaria vector populations but was found in the data from Ae. aegypti populations, although caution is needed when interpreting differences found using susceptibility test data (particularly tests using diagnostic doses that have not been calibrated for Aedes species [34]). Etofenprox is the active ingredient in two WHO prequalified products; a kit for insecticide-treated nets (Vectron 10EW) and an IRS formulation (Vectron20WP) [59]. The latter product is listed by the Global Fund, but etofenprox is not widely deployed in Africa and was last reported as the active ingredient used for IRS in 2012 in parts of Zambia [51, 52].

We found some variation in the relationships among pyrethroids when different types of evidence were considered. In particular, the results for insecticide depletion were largely not repeated in the findings for resistance in mosquito populations. The results for both insecticide inhibition and insecticide depletion depend on which enzymes are included in the activity tests. Seven P450s (three for the depletion analysis) were included here whereas at least 14 have been implicated in An. gambiae s.l. and An. funestus resistance so far [21, 22, 24–32, 46, 60–74] and many more in Aedes vectors [34]. It is also important to note that detoxification by P450s is not the only mechanism of resistance found in these vector species. Target site mutations are common in many of these species [9–13], upregulation of other detoxifying enzymes is also linked to pyrethroid resistance [75] and there is some evidence for cuticular thickening in resistant mosquitoes [76]. Upregulation of the GSTE2 gene is associated with resistance to both permethrin and deltamethrin, as well as DDT, in An. gambiae and An. coluzzii [71, 73, 77], An. funestus [29, 72, 75] and Ae. aegypti [78–80], and allele frequencies for target site mutations in the voltage-gated sodium channel gene, Vgsc, have been shown to be useful partial predictors of resistance in An. gambiae s.l. [35]. Thus, we would not expect the findings from molecular studies of P450 activity alone to be exactly replicated in field populations, except in instances where P450-mediated metabolic resistance dominates in a mosquito population.

The results for pyrethroid cross-resistance within individual species reported here match our knowledge of other mechanisms of resistance found in these species. Mutations in the Vgsc gene (kdr mutations) confer cross-resistance to pyrethroids and DDT, and are partial predictors of patterns of resistance to these compounds in the An. gambiae complex, but have not been found in An. funestus or other members of the An. funestus subgroup [3, 8–14, 35]. In our study, correlations between pyrethroid and
DDT resistance were found for members of the *An. gambiae* complex but not for the *An. funestus* subgroup or species. No correlations were found between pyrethroid resistance and resistance to the carbamates or organochlorines, underlining the finding that it is cross-resistance within the pyrethroids, as well as between the pyrethroids and DDT, that is most important. Some metabolic resistance mechanisms do confer cross-class resistance, e.g. between the pyrethroids and DDT and/or the carbamates [24, 30, 32, 74], but the impact of these mechanisms within the array of resistance types that co-occur is more nuanced, and no cross-class resistance other than the aforementioned pyrethroid-DDT resistance in *An. gambiae* s.l. was detected here.

In conclusion, we have found that evidence for cross-resistance among pyrethroids predicted by SAR studies of metabolic resistance can be detected across African mosquito populations, as exemplified by i) the close associations between the binding affinities of permethrin and deltamethrin to a range of anopheline P450s, ii) the close associations between depletion of permethrin and deltamethrin by these P450s, and iii) correlations in resistance to permethrin and deltamethrin in populations of *An. arabiensis*, *An. coluzzii*, *An. gambiae* and *An. funestus*. Importantly, populations with higher resistance to one of the pyrethroids studied here, which all contain the common structural motif of phenoxy benzyl alcohol coupled with a cyclopropane ring (the primary target for metabolic oxidation), are likely to have higher resistance to the others and these cross-resistance trends could be detected despite the noise in these susceptibility test data. It is unlikely that resistance to those pyrethroids most commonly deployed for malaria control diverges within vector populations and it would be unwise to switch between these compounds based on the results from a small number of susceptibility tests alone. There are, however, pyrethroids that are not commonly deployed that show greater potential for true divergence in resistance, such as bifenthrin and possibly etofenprox. It is worth noting that there are still significant correlations between resistance to etofenprox and resistance to the pyrethroids in common use, and that this is largely untested for bifenthrin. Systematic SAR analyses of these more structurally diverse pyrethroids are required to estimate the affect of structural diversity on pyrethroid resistance and these findings need to be verified by studies of resistance in wild populations.

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

All authors provided their consent for publication.

**Availability of data and material**

All susceptibility test data analysed during this study are included in Additional File 4.

**Competing interests**
The authors confirm they have no competing interests.

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**Authors’ contributions**

CLM and HMI conceived the study. CLM, CY, KJW, KH, FO, MJIP and HMI generated the data and conducted the analyses. All authors contributed to the interpretation of the results and preparation of the manuscript.

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**References**

1. World Health Organization: World Malaria Report 2019. Geneva 2019.
2. World Health Organization: Prequalified Lists. [https://www.who.int/pq-vector-control/prequalified-lists/en/] (2020). Accessed 11 August 2020.
3. Hancock PA, Hendriks CJM, Tangena JA, Gibson H, Hemingway J, Coleman M, et al. Mapping trends in insecticide resistance phenotypes in African malaria vectors. Plos Biology. 2020;18 6; doi: 10.1371/journal.pbio.3000633.
4. Moyes CL, Athinya DK, Seethaler T, Battle KE, Sinka M, Hadi MP, et al. Evaluating insecticide resistance across African districts to aid malaria control decisions. Proceedings of the National Academy of Sciences of the United States of America. 2020;117 36:22042-50; doi: 10.1073/pnas.2006781117.
5. Soderlund DM. Molecular mechanisms of pyrethroid insecticide neurotoxicity: recent advances. Arch Toxicol. 2012;86 2:165-81; doi: 10.1007/s00204-011-0726-x.
6. Narahashi T. Nerve membrane ionic channels as the primary target of pyrethroids. Neurotoxicology. 1985;6 2:3-22
7. Sinka ME, Golding N, Massey NC, Wiebe A, Huang Z, Hay SI, et al. Modelling the relative abundance of the primary African vectors of malaria before and after the implementation of indoor, insecticide-based vector control. Malaria Journal. 2016;15; doi: 10.1186/s12936-016-1187-8.
8. Kawada H, Dida GO, Ohashi K, Komagata O, Kasai S, Tomita T, et al. Multimodal Pyrethroid Resistance in Malaria Vectors, Anopheles gambiae s.s., Anopheles arabiensis, and Anopheles funestus s.s. in Western Kenya. Plos One. 2011;6 8; doi: 10.1371/journal.pone.0022574.
9. Camara S, Koffi AA, Alou LPA, Koffi K, Kabran JPK, Kone A, et al. Mapping insecticide resistance in Anopheles gambiae (s.l.) from Cote d’Ivoire. Parasites & Vectors. 2018;11; doi: 10.1186/s13071-017-2546-1.

10. Foster GM, Coleman M, Thomsen E, Ranson H, Yangalbe-Kalnone E, Moundai T, et al. Spatial and Temporal Trends in Insecticide Resistance among Malaria Vectors in Chad Highlight the Importance of Continual Monitoring. Plos One. 2016;11 5; doi: 10.1371/journal.pone.0155746.

11. Kawada H, Futami K, Komagata O, Kasai S, Tomita T, Sonye G, et al. Distribution of a Knockdown Resistance Mutation (L1014S) in Anopheles gambiae s.s. and Anopheles arabiensis in Western and Southern Kenya. Plos One. 2011;6 9; doi: 10.1371/journal.pone.0024323.

12. Ndiath MO, Cailleau A, Orlandi-Pradines E, Bessell P, Pages F, Trape JF, et al. Emerging knock-down resistance in Anopheles arabiensis populations of Dakar, Senegal: first evidence of a high prevalence of kdr-e mutation in West African urban area. Malaria Journal. 2015;14; doi: 10.1186/s12936-015-0898-6.

13. Reddy MR, Godoy A, Dion K, Matias A, Callender K, Kiszewski AE, et al. Insecticide Resistance Allele Frequencies in Anopheles gambiae before and after Anti-Vector Interventions in Continental Equatorial Guinea. American Journal of Tropical Medicine and Hygiene. 2013;88 5:897-907; doi: 10.4269/ajtmh.12-0467.

14. Moyes CL, Wiebe A, Gleave K, Trett A, Hancock PA, Padonou GG, et al. Analysis-ready datasets for insecticide resistance phenotype and genotype frequency in African malaria vectors. Scientific Data. 2019;6; doi: 10.1038/s41597-019-0134-2.

15. Rinkevich FD, Du Y, Dong K. Diversity and Convergence of Sodium Channel Mutations Involved in Resistance to Pyrethroids. Pestic Biochem Physiol. 2013;106 3:93-100; doi: 10.1016/j.pestbp.2013.02.007.

16. Khambay BPS, Farnham, A.W., Beddie, D.G. Relationships between pyrethroid structure and level of resistance in houseflies (Musca domestica L.). Advances in the Chemistry of Insect Control III. 1994;Pages 117-126

17. Davies TGE, Williamson M. Interactions of pyrethroids with the voltage-gated sodium channel. Bayer Crop Sci J. 2009;62

18. Awolola TS, Adeogun A, Olakiigbe AK, Oyeniyi T, Olukosi YA, Okoh H, et al. Pyrethroids resistance intensity and resistance mechanisms in Anopheles gambiae from malaria vector surveillance sites in Nigeria. Plos One. 2018;13 12; doi: 10.1371/journal.pone.0205230.

19. Hemingway J, Vontas J, Poupardin R, Raman J, Lines J, Schwabe C, et al. Country-level operational implementation of the Global Plan for Insecticide Resistance Management. Proceedings of the National Academy of Sciences of the United States of America. 2013;110 23:9397-402; doi: 10.1073/pnas.1307656110.

20. Irving H, Wondji CS. Investigating knockdown resistance (kdr) mechanism against pyrethroids/DDT in the malaria vector Anopheles funestus across Africa. Bmc Genetics. 2017;18; doi: 10.1186/s12863-017-0539-x.
21. David JP, Ismail HM, Chandor-Proust A, Paine MJ. Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on Earth. Philosophical Transactions of the Royal Society B-Biological Sciences. 2013;368 1612; doi: 10.1098/rstb.2012.0429.

22. Amenya DA, Naguran R, Lo TCM, Ranson H, Spillings BL, Wood OR, et al. Over expression of a cytochrome p450 (CYP6P9) in a major African malaria vector, Anopheles funestus, resistant to pyrethroids. Insect Molecular Biology. 2008;17 1:19-25; doi: 10.1111/j.1365-2583.2008.00776.x.

23. Barnes KG, Irving H, Chiumia M, Mzilahowa T, Coleman M, Hemingway J, et al. Restriction to gene flow is associated with changes in the molecular basis of pyrethroid resistance in the malaria vector Anopheles funestus. Proceedings of the National Academy of Sciences of the United States of America. 2017;114 2:286-91; doi: 10.1073/pnas.1615458114.

24. Kwiatkowska RM, Platt N, Poupardin R, Irving H, Dabire RK, Mitchell S, et al. Dissecting the mechanisms responsible for the multiple insecticide resistance phenotype in Anopheles gambiae s.s., M form, from Vallee du Kou, Burkina Faso. Gene. 2013;519 1:98-106; doi: 10.1016/j.gene.2013.01.036.

25. Muller P, Donnelly MJ, Ranson H. Transcription profiling of a recently colonised pyrethroid resistant Anopheles gambiae strain from Ghana. Bmc Genomics. 2007;8; doi: 10.1186/1471-2164-8-36.

26. Ngufor C, N'Guessan R, Fagbohoun J, Subramaniam K, Odjo A, Fongnikin A, et al. Insecticide resistance profile of Anopheles gambiae from a phase II field station in Cove, southern Benin: implications for the evaluation of novel vector control products. Malaria Journal. 2015;14; doi: 10.1186/s12936-015-0981-z.

27. Nikou D, Ranson H, Hemingway J. An adult-specific CYP6P450 gene is overexpressed in a pyrethroid-resistant strain of the malaria vector, Anopheles gambiae. Gene. 2003;318:91-102; doi: 10.1016/s0378-1119(03)00763-7.

28. Riveron JM, Ibrahim SS, Chanda E, Mzilahowa T, Cuamba N, Irving H, et al. The highly polymorphic CYP6M7 cytochrome P450 gene partners with the directionally selected CYP6P9a and CYP6P9b genes to expand the pyrethroid resistance front in the malaria vector Anopheles funestus in Africa. Bmc Genomics. 2014;15; doi: 10.1186/1471-2164-15-817.

29. Riveron JM, Irving H, Ndula M, Barnes KG, Ibrahim SS, Paine MJ, et al. Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector Anopheles funestus. Proceedings of the National Academy of Sciences of the United States of America. 2013;110 1:252-7; doi: 10.1073/pnas.1216705110.

30. Thomsen EK, Strode C, Hemmings K, Hughes AJ, Chanda E, Musapa M, et al. Underpinning sustainable vector control through informed insecticide resistance management. Plos One. 2014;9 6; doi: 10.1371/journal.pone.0099822.

31. Vontas J, Grigoraki L, Morgan J, Tsakireli D, Fuseini G, Segura L, et al. Rapid selection of a pyrethroid metabolic enzyme CYP9K1 by operational malaria control activities. Proceedings of the National
32. Ibrahim SS, Ndula M, Riveron JM, Irving H, Wondji CS. The P450 CYP6Z1 confers carbamate/pyrethroid cross-resistance in a major African malaria vector beside a novel carbamate-insensitive N485I acetylcholinesterase-1 mutation. Molecular Ecology. 2016;25 14:3436-52; doi: 10.1111/mec.13673.

33. Weetman D, Kamgang B, Badolo A, Moyes CL, Shearer FM, Coulibaly M, et al. Aedes Mosquitoes and Aedes-Borne Arboviruses in Africa: Current and Future Threats. International Journal of Environmental Research and Public Health. 2018;15 2; doi: 10.3390/ijerph15020220.

34. Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I, et al. Contemporary status of insecticide resistance in the major Aedes vectors of arboviruses infecting humans. Plos Neglected Tropical Diseases. 2017;11 7; doi: 10.1371/journal.pntd.0005625.

35. Hancock PA, Wiebe A, Gleave KA, Bhatt S, Cameron E, Trett A, et al. Associated patterns of insecticide resistance in field populations of malaria vectors across Africa. Proceedings of the National Academy of Sciences of the United States of America. 2018;115 23:5938-43; doi: 10.1073/pnas.1801826115.

36. World Health Organization: Vector resistance to pesticides: Fifteenth report of the WHO expert committee on vector biology and control. Geneva1992: 68.

37. World Health Organization: Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy nd persistance of insecticides on treated surfaces. Geneva1998: 46.

38. Yunta C, Hemmings K, Stevenson B, Koekemoer LL, Matambo T, Pignatelli P, et al. Cross-resistance profiles of malaria mosquito P450s associated with pyrethroid resistance against WHO insecticides. Pesticide Biochemistry and Physiology. 2019;161:61-7; doi: 10.1016/j.pestbp.2019.06.007.

39. Kalliokoski T, Kramer C, Vulpetti A, Gedeck P. Comparability of Mixed IC50 Data - A Statistical Analysis. Plos One. 2013;8 4; doi: 10.1371/journal.pone.0061007.

40. McLaughlin LA, Niazi U, Bibby J, David JP, Vontas J, Hemingway J, et al. Characterization of inhibitors and substrates of Anopheles gambiae CYP6Z2. Insect Molecular Biology. 2008;17 2:125-35; doi: 10.1111/j.1365-2583.2007.00788.x.

41. Yunta C, Grisales N, Nasz S, Hemmings K, Pignatelli P, Voice M, et al. Pyriproxyfen is metabolized by P450s associated with pyrethroid resistance in An. gambiae. Insect Biochemistry and Molecular Biology. 2016;78:50-7; doi: 10.1016/j.ibmb.2016.09.001.

42. Holm S. A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics. 1979;6 2:65-70

43. Sokal RR, Michener CD. A statistical method for evaluating systematic relationships. University of Kansas Science Bulletin. 1958;38:1409-38

44. Krippendorff BF, Lienau P, Reichel A, Huisinga W. Optimizing classification of drug-drug interaction potential for CYP450 isoenzyme inhibition assays in early drug discovery. Journal of Biomolecular Screening. 2007;12 1:92-9; doi: 10.1177/1087057106295897.
45. Muller P, Warr E, Stevenson BJ, Pignatelli PM, Morgan JC, Steven A, et al. Field-caught permethrin-resistant Anopheles gambiae overexpress CYP6P3, a P450 that metabolises pyrethroids. PLoS Genet. 2008;4 11:e1000286; doi: 10.1371/journal.pgen.1000286.

46. Stevenson BJ, Bibby J, Pignatelli P, Muangnoicharoen S, O'Neill PM, Lian LY, et al. Cytochrome P450 6M2 from the malaria vector Anopheles gambiae metabolizes pyrethroids: Sequential metabolism of deltamethrin revealed. Insect Biochemistry and Molecular Biology. 2011;41 7:492-502; doi: 10.1016/j.ibmb.2011.02.003.

47. Horstmann S, Sonneck R. Contact Bioassays with Phenoxybenzyl and Tetrafluorobenzyl Pyrethroids against Target-Site and Metabolic Resistant Mosquitoes. PLoS One. 2016;11 3:e0149738; doi: 10.1371/journal.pone.0149738.

48. Tan JG, McCaffery AR. Efficacy of various pyrethroid structures against a highly metabolically resistant isogenic strain of Helicoverpa armigera (Lepidoptera : Noctuidae) from China. Pest Management Science. 2007;63 10:960-8; doi: 10.1002/ps.1419.

49. The Global Fund: List of indoor residual sprays (IRS) that meet GF QA requirements for use against malaria vector. 
https://www.theglobalfund.org/media/5857/psm_indoorresidualsprayirsgf_list_en.pdf?u=636679306830000000 (2020). Accessed 12 August 2020.

50. Okwa OO. The current trends in integrated prevention and control of malaria. A case study of some Nigerian communities Global Advanced Resarch Journal of Medicine and Medical Sciences. 2012;2:104-7

51. Tangena JAA, Hendriks CMJ, Devine M, Tammaro M, Trett AE, Williams I, et al. Indoor residual spraying for malaria control in sub-Saharan Africa 1997 to 2017: an adjusted retrospective analysis. Malaria Journal. 2020;19 1; doi: 10.1186/s12936-020-03216-6.

52. Chanda E, Kandyata A, Chanda J, Phiri FN, Muzia L, Kamuliwo M. The efficacy of Vectron 20 WP, etofenprox, for indoor residual spraying in areas of high vector resistance to pyrethroids and organochlorines in Zambia. International Scholarly Research Notes. 2012;2013:e371934

53. Ozumba N, Onyido A, Nwosu E, Ekwunife E, Amadi E. Field trial tests on the efficacy and residual effects of Bistar® 10wp on mosquitoes and other household arthropod pests. The Internet Journal of Tropical Medicine. 2008;6

54. RTI International: Rwanda Spraying Performance Report. 2007.

55. Chang KS, Yoo DH, Shin EH, Lee WG, Roh JY, Park MY. Susceptibility and resistance of field populations of Anopheles sinensis (Diptera: Culicidae) collected from Paju to 13 insecticides. Osong Public Health Res Perspect. 2013;4 2:76-80; doi: 10.1016/j.phrp.2013.02.001.

56. Flores AE, Ponce G, Silva BG, Gutierrez SM, Bobadilla C, Lopez B, et al. Wide spread cross resistance to pyrethroids in Aedes aegypti (Diptera: Culicidae) from Veracruz State Mexico. Journal of Economic Entomology. 2013;106 2:959-69; doi: 10.1603/ec12284.

57. Juntarajumnong W, Pimnon S, Bangs MJ, Thanispong K, Chareonviriyaphap T. Discriminating lethal concentrations and efficacy of six pyrethroids for control of Aedes aegypti in Thailand. Journal of
58. Thanispong K, Sathantriphop S, Malaitthong N, Bangs MJ, Charoenviriyaphap T. Establishment of diagnostic doses of five pyrethroids for monitoring physiological resistance in *Aedes albopictus* in Thailand. Journal of the American Mosquito Control Association. 2015;31 4:346-52

59. WHO. Prequalification Vector Control, Prequalified Lists. World Health Organisation 2020;

60. Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, et al. Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae* s.s. from Southern Benin and Nigeria. Bmc Genomics. 2008;9; doi: 10.1186/1471-2164-9-538.

61. Munhenga G, Koekemoer LL. Differential expression of cytochrome P450 genes in a laboratory selected *Anopheles arabiensis* colony. African Journal of Biotechnology. 2011;10 59; doi: 10.5897/ajb11.363.

62. Nardini L, Christian RN, Coetzer N, Koekemoer LL. DDT and pyrethroid resistance in *Anopheles arabiensis* from South Africa. Parasites & Vectors. 2013;6; doi: 10.1186/1756-3305-6-229.

63. Jones CM, Haji KA, Khatib BO, Bagi J, Mcha J, Devine GJ, et al. The dynamics of pyrethroid resistance in *Anopheles arabiensis* from Zanzibar and an assessment of the underlying genetic basis. Parasites & Vectors. 2013;6; doi: 10.1186/1756-3305-6-343.

64. Abdalla H, Wilding CS, Nardini L, Pignatelli P, Koekemoer LL, Ranson H, et al. Insecticide resistance in *Anopheles arabiensis* in Sudan: temporal trends and underlying mechanisms. Parasites & Vectors. 2014;7; doi: 10.1186/1756-3305-7-213.

65. Main BJ. Complex and evolving insecticide resistance spans species barriers in *Anopheles culuzii*. American Journal of Tropical Medicine and Hygiene. 2015;93 4:363-4

66. Main BJ, Lee Y, Collier TC, Norris LC, Brisco K, Fofana A, et al. Complex genome evolution in *Anopheles culuzii* associated with increased insecticide usage in Mali. Molecular Ecology. 2015;24 20:5145-57; doi: 10.1111/mec.13382.

67. Ibrahim SS, Riveron JM, Stott R, Irving H, Wondji CS. The cytochrome P450 CYP6P4 is responsible for the high pyrethroid resistance in knockdown resistance-free *Anopheles arabiensis*. Insect Biochemistry and Molecular Biology. 2016;68:23-32; doi: 10.1016/j.ibmb.2015.10.015.

68. Lucas ER, Rockett KA, Lynd A, Essandoh J, Grisales N, Kemei B, et al. A high throughput multi-locus insecticide resistance marker panel for tracking resistance emergence and spread in *Anopheles gambiae*. Scientific Reports. 2019;9; doi: 10.1038/s41598-019-49892-6.

69. Ibrahim SS, Amvongo-Adjia N, Wondji MJ, Irving H, Riveron JM, Wondji CS. Pyrethroid resistance in the major malaria vector *Anopheles funestus* is exacerbated by overexpression and overactivity of the P450 CYP6AA1 across Africa. Genes. 2018;9 3; doi: 10.3390/genes9030140.

70. Barnes KG, Weedall GD, Ndula M, Irving H, Mzihalowa T, Hemingway J, et al. Genomic footprints of selective sweeps from metabolic resistance to pyrethroids in African malaria vectors are driven by scale up of insecticide-based vector control. Plos Genetics. 2017;13 2; doi: 10.1371/journal.pgen.1006539.
71. Toe KH, N’Fale S, Dabire RK, Ranson H, Jones CM. The recent escalation in strength of pyrethroid resistance in *Anopheles coluzzi* in West Africa is linked to increased expression of multiple gene families. Bmc Genomics. 2015;16; doi: 10.1186/s12864-015-1342-6.

72. Gregory R, Darby AC, Irving H, Coulibaly MB, Hughes M, Koekemoer LL, et al. A de novo expression profiling of *Anopheles funestus*, malaria vector in Africa, using 454 pyrosequencing. Plos One. 2011;6 2; doi: 10.1371/journal.pone.0017418.

73. David JP, Strode C, Vontas J, Nikou D, Vaughan A, Pignatelli PM, et al. The *Anopheles gambiae* detoxication chip: A highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. Proceedings of the National Academy of Sciences of the United States of America. 2005;102 11:4080-4; doi: 10.1073/pnas.0409348102.

74. Edi CV, Djogbenou L, Jenkins AM, Regna K, Muskavitch MAT, Poupardin R, et al. CYP6 P450 enzymes and ACE-1 duplication produce extreme and multiple insecticide resistance in the malaria mosquito *Anopheles gambiae*. Plos Genetics. 2014;10 3; doi: 10.1371/journal.pgen.1004236.

75. Riveron JM, Yunta C, Ibrahim SS, Djouaka R, Irving H, Menze BD, et al. A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. Genome Biology. 2014;15 2; doi: 10.1186/gb-2014-15-2-r27.

76. Balabanidou V, Kefi M, Aivaliotis M, Koidou V, Girotti JR, Mijailovsky SJ, et al. Mosquitoes cloak their legs to resist insecticides. Proceedings of the Royal Society B-Biological Sciences. 2019;286 1907; doi: 10.1098/rspb.2019.1091.

77. Ding YC, Ortelli F, Rossiter LC, Hemingway J, Ranson H. The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. Bmc Genomics. 2003;4; doi: 10.1186/1471-2164-4-35.

78. Lumjuan N, Rajatileka S, Changsom D, Wicheer J, Leelapat P, Prapanthadara LA, et al. The role of the *Aedes aegypti* Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides. Insect Biochemistry and Molecular Biology. 2011;41 3:203-9; doi: 10.1016/j.ibmb.2010.12.005.

79. Strode C, Wondji CS, David JP, Hawkes NJ, Lumjuan N, Nelson DR, et al. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. Insect Biochemistry and Molecular Biology. 2008;38 1:113-23; doi: 10.1016/j.ibmb.2007.09.007.

80. Lumjuan N, McCarroll L, Prapanthadara LA, Hemingway J, Ranson H. Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti*. Insect Biochemistry and Molecular Biology. 2005;35 8:861-71; doi: 10.1016/j.ibmb.2005.03.008.

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**Additional File 4**

Additional File 4 was not provided with this version of the manuscript.

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**Figures**
Figure 1

Chemical structure of pyrethroid insecticides used for malaria vector control. The common scaffold of pyrethroids, boxed in red, was identified by searching 230 million compounds available in the ZINC database (https://zinc.docking.org).

X Y Z

α-cypermethrin Cl Cl H
Cyfluthrin Cl Cl F
λ-cyhalothrin Cl CF₃ H
Deltamethrin Br Br H
Chemical structure of pyrethroid insecticides used for malaria vector control. The common scaffold of pyrethroids, boxed in red, was identified by searching 230 million compounds available in the ZINC database (https://zinc.docking.org).

Figure 2

Cluster analysis of functional activity data for six pyrethroids against P450s from African malaria vectors. (A) Inhibition data from the screening of six pyrethroids (scaffold structures indicated on the right of data panels) against a set of P450s are presented as a heat map. Target enzymes are arrayed along the x-axis, and each of the pyrethroids is arrayed along the y-axis. Colours indicate the inhibition potency of pyrethroids with an indicated variable scaffold for a designated target P450. Potent (hot) inhibitors are assigned a red colour, and weak or ineffective (cold) inhibitors are given a light green colour. (B) Pyrethroid metabolism by the P450s most widely associated with resistance from An. gambiae (CYP6M2 and CYP6P3) and An. funestus (CYP6P9a) is clustered and presented as a heat map. Pyrethroids susceptible to metabolism are assigned a red colour, and weak metabolism denoted light green. Dendrograms were obtained by hierarchical clustering. They indicate the degree of similarity as a function of the height of the lines connecting the profiles.
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Figure 3

The distributions of values for three example pyrethroid pairs (A) the most closely related pyrethroid pair in terms of resistance in wild mosquito populations (deltamethrin and \(\lambda\)-cyhalothrin), (B) a mid-ranked pair (permethrin and \(\alpha\)-cypermethrin) and (C) the least closely related pair (\(\lambda\)-cyhalothrin and etofenprox). Each point represents the results from a single An. gambiae s.l. sample that was subdivided between two susceptibility tests. The full results for every pair are shown in Figure S2, Additional File 2.
Figure 4

Mean correlation coefficients for resistance to pairs of pyrethroids in An. gambiae s.l. ‘alph.’ is α-cypermethrin, ‘cyfl.’ is cyfluthrin, ‘delt.’ is deltamethrin, ‘etof.’ is etofenprox, ‘lamb.’ is λ-cyhalothrin and ‘perm.’ is permethrin. The bars represent the upper and lower 95% bootstrap confidence intervals and the sample size for each pair is given below these bars.
Figure 4

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Figure 5

Hierarchical relationships among pyrethroids measured using data on resistance in vectors and functional activity data. The dendrograms were constructed using (A) correlations in mortality across
African malaria vector populations (Pearson’s correlation coefficient), (B) insecticide depletion values (%), and (C) binding affinity values (IC50).

Figure 5

Hierarchical relationships among pyrethroids measured using data on resistance in vectors and functional activity data. The dendrograms were constructed using (A) correlations in mortality across African malaria vector populations (Pearson’s correlation coefficient), (B) insecticide depletion values (%), and (C) binding affinity values (IC50).

Supplementary Files

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