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Reviewing the evidence for and against selection of specific pyrethroids for programmatic purposes

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Acronym list

CDC	Centre for Disease Control
CI	Credible interval
CYP	Cytochrome P450
DD	Diagnostic doses
GABA	gamma-aminobutyric acid receptor
GST	Glutathione-S-transferase
I-ACT	Ifakara ambient chamber test
IRS	Indoor residual spraying
ITN	Insecticide treated net
KC	Knockdown data
Kdr	Knockdown resistance
LC	Lethal concentration
LD	Lethal dose
LITE	Liverpool Insecticide Testing Establishment
LMC	Linear model of coregionalization
LSTM	Liverpool School of Tropical Medicine
MCD	Mosquito contamination device
P450	Cytochrome P450
PBO	Piperonyl butoxide
PMI	President's Malaria Initiative

QSAR	Quantitative structure-activity relationship
RRKC	Resistance ratio knockdown data
RRLC	Resistance ratio lethal concentrations
RS	Relative susceptibility
SAR	structure-activity relationship
s-kdr	Super-knockdown
UPGMA	Unweighted pair-group method with arithmetic mean
VCT	Video cone test
VGSC	Voltage-gated sodium channel
ViCTA	Video cone test analysis
WHO	World Health Organization

1. Introduction

Pyrethroid resistance is widespread in malaria vectors. Pyrethroids are present in all WHO prequalified insecticide treated nets (ITNs) and are still used for indoor residual spraying (IRS). Where susceptibility monitoring suggests differential levels of resistance to different pyrethroids, PMI, a key vector control procurement agency, supports the choice of products containing pyrethroid active ingredients which show higher mortality in bioassay tests against the local mosquito population. However, there is some uncertainty about whether current methods for susceptibility monitoring can reliably identify differential resistance phenotypes, and if so, whether effective resistance management can be achieved by such targeted use of insecticides of the same class. This desk review aims to answer the following two main questions:

1. Should countries interpret differential mortality in discriminating dose susceptibility assays as indication of differential levels of susceptibility within the pyrethroid class? Or should these be interpreted in another way (e.g. inherent variability in mortality results; differently calibrated discriminating doses, other).

If yes:

2. Should countries with evidence of differential susceptibility in pyrethroid assays consider preferentially selecting a specific insecticide for programmatic use? i.e. does a difference in mortality in a diagnostic dose or resistance intensity bioassay imply either different control potential or potential to use multiple pyrethroids in resistance management approaches?

Evidence from the following areas were examined: i) molecular information ii) insecticide resistance patterns and testing results in laboratory colonies iii) insecticide resistance patterns and testing results in field data iv) lessons from behavioural assays.

Main questions and the sources of evidence used to address them

Molecular evidence

1. Is there molecular evidence for differential resistance among members of the pyrethroid insecticide class?
2. Are different pyrethroids equally susceptible to different resistance mechanisms?

Laboratory strains

Using discriminating dose assays on laboratory reared colonies to ask:

3. Are the discriminating doses of permethrin and deltamethrin suitable and comparable?
4. What intrinsic variability do we see in the results of dose response assays?
5. Is there evidence for divergent resistance in colonies routinely selected using a single pyrethroid?

Field populations

Using data from discriminating dose and intensity assays on field populations:

6. What are potential sources of (non-resistance associated) variability in the assay?
7. What is the evidence for the existence of divergent resistance between pyrethroids?
8. Can difference seen in molecular studies (question 1) be detected in wild mosquito populations?

Mosquito behaviour

9. Do mosquitoes, resistant or susceptible, exhibit different behavioural responses to different pyrethroids?
10. How suitable are resistance monitoring methods considering the possibility of behavioural resistance?
11. How could efficacy testing be improved to take behavioural response into account?

2. Molecular evidence

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2.1. Background

Pyrethroids continue to dominate the vector control market because of their high insecticidal activity against mosquitoes and their low toxicity to mammals. Sixty-seven per cent of the WHO prequalified vector control products contain pyrethroids alone or in combination with other chemistries (Table 2.1) (WHO, 2020). Synthetic pyrethroids are derivatives of pyrethrins, natural pesticide chemicals found in flowerheads of two species of asters: *Chrysanthemum cinerariifolium* and *C. coccineum*. Whilst pyrethrins are effective in killing insects, they rapidly degrade in sunlight. To overcome these limitations, numerous synthetic pyrethroids have been developed using pyrethrins as a chemical template (Casida, 1980). Pyrethroids work by binding with the voltage-gated sodium channel (VGSC) in the insect neurons, which results in depolarization caused by the prolonged influx of sodium ions during excitation. The extended depolarisation resulting from pyrethroid binding is what leads to repetitive nerve activity that can result in knockdown and death (Narahashi, 1985; Soderlund, 2012). Pyrethroids, which comprise a diverse range of structures, are historically differentiated into two broad groups based on their biological activity that is associated with the absence (Type I) or presence (Type II) of an α-cyano group. Type II pyrethroids are more lethal to insects because of their higher affinity to the VGSC in the nerve membrane. They also interact with other insect targets such as gamma-aminobutyric acid receptor (GABA) and inhibit ATPase activity (Narahashi, 1985; Soderlund, 2012). The higher lethality of Type II pyrethroids, such as deltamethrin and α-cypermethrin, translates into lower doses in field products compared with Type I pyrethroids, such as permethrin, and this has led to abundant use of α-cyano pyrethroids in WHO pre-qualified products (Table 1) (WHO, 2020).

*Table 2.1. Summary of WHO's prequalified vector control products (January 2020). Abbreviations: IRS = Indoor residual spraying, LLIN = Long-lasting insecticide treated net, ITN = Insecticide treated net. *Not recommended by WHO as an intervention for malaria control.*

Intervention type	Products	Pyrethroid	Chemicals	Combinations
IRS	24	19	(9) α-cypermethrin, (4) deltamethrin, (4) λ-cyhalothrin, etofenprox and bifenthrin	Fludora Fusion (clothianidin; deltamethrin)
LLIN	20	20	(2) permethrin, (10) α-cypermethrin & (8) deltamethrin	Interceptor G2 (α-cypermethrin; chlorfenapyr) & Royal Guard (α-cypermethrin; pyriproxyfen)
ITN kit	7	7	(4) α-cypermethrin, λ-cyhalothrin, etofenprox	
Space Spraying*	10	8	trans-cyphenothrin, prallethrin, S-bioallethrin, (3) deltamethrin, λ-cyhalothrin and permethrin	Cielo ULV (prallethrin; imidacloprid) & Aqua Reslin Super (S-bioallethrin (esdepallethrine), permethrin and piperonyl Butoxide)
Larvicide	20	0		
Total	81	54		

Pyrethroids used in vector control generally possess the common structural motif of phenoxy benzyl alcohol coupled with a cyclopropane ring via an ester bond, except for the Type I insecticides bifenthrin and etofenprox (Appendix 1. Figure S2.1). This limited chemical variability within the insecticide class increases the chances of cross-resistance, which would thus decrease the opportunity for different pyrethroids to be used together in effective insecticide resistance management. However, our understanding of the impact of this cross-resistance mechanism is limited, and a comprehensive review of this will help inform the best use of different compounds in the field and avoid the costly use of chemistries that are unlikely to work.

Pyrethroid resistance in the *An. gambiae* complex is primarily associated with target-site insensitivity due to mutations in the VGSC gene that cause knockdown resistance (*kdr*), and metabolic resistance from increased insecticide detoxification (David *et al.*, 2013). *Kdr* mutations are found at high frequencies (sometimes 100%) in the major African malaria vectors, *An. gambiae* and *An. coluzzii* (Fadel *et al.*, 2019), but are absent in *An. funestus*, where metabolic resistance mechanisms dominate (Irving and Wondji, 2017). Whole genome sequencing of multiple *An. gambiae* populations has

detected many amino acid substitutions in the VGSC gene, especially in west Africa (Clarkson *et al.*, 2018), but currently only three of these mutations have been proven to cause pyrethroid resistance in *Anopheles* mosquitoes; L995F, L995S, and N1570Y (Donnelly, Isaacs and Weetman, 2016). L995F (L1014F in *Musca domesticus* codon numbering) is prevalent in west and central Africa, L995S is found in central and east Africa, and N1570Y is found in west and central Africa, amplifying the resistance conferred by 995F to permethrin and deltamethrin (C. M. Jones *et al.*, 2012). There are, however, a large variety of additional amino acid substitutions found in other insects that produce a resistance phenotype (Rinkevich, Du and Dong, 2013), for example M918T, a mutation that produces a super-knockdown (s-*kdr*) phenotype in houseflies. Structural modelling studies indicate that the degree of resistance in s-*kdr* houseflies depends on the chemical structure of the pyrethroid. Resistance is highly correlated with the presence of α-cyano group coupled with phenoxybenzyl moiety in the larger Type II pyrethroid molecules such as deltamethrin and fenvalerate (Khambay, Farnham and Beddie, 1994). By comparison, the most common VGSC resistance allele in west African *An. gambiae* populations, L1014F (L995F), is not influenced by pyrethroid chemical structure when expressed alone in house flies (Davies and Williamson, 2009).

Phenotypic bioassay data suggest some differences in the association between *An. gambiae kdr* 995 mutations with permethrin and deltamethrin survivorship, with significantly lower predictive value for the latter (Reimer *et al.*, 2008; Ramphul *et al.*, 2009). Yet these differences are slight when compared to the much stronger effects of *kdr* 995 mutations on DDT survival (Reimer *et al.*, 2008; Ramphul *et al.*, 2009; Opondo *et al.*, 2016). Electrophysiological studies have detected little difference in effects on permethrin or deltamethrin binding for a range of *kdr* mutations including those at the 995 codon (Rinkevich, Du and Dong, 2013) or 995F and 1570Y combined (Wang *et al.*, 2015) (Li *et al.*, 2015). Similarly, houseflies with different *kdr* mutations (including L1014F) tested against a very broad range of pyrethroids showed markedly similar resistance profiles to cypermethrin, permethrin and deltamethrin, the three most operationally important for malaria control; however orders of magnitude difference were observed between structurally-divergent compounds such as etofenprox and transfluthrin (Sun *et al.*, 2016). Overall, there is little evidence to support differential effects of currently widespread *Anopheles* target site mutations among currently commonly used pyrethroids, but further empirical studies may be required if a greater range of pyrethroids are considered for operational use.

Whether alone or in combination *kdr* mutations cannot fully explain the high levels of pyrethroid resistance observed in contemporary African *Anopheles* malaria vectors and other mechanisms are

typically implicated. For example, reduced susceptibility to pyrethroids in *An. gambiae* is often due to a combination of *kdr* alleles and multigenic metabolic resistance mechanisms (Stica *et al.*, 2019). By contrast, the low susceptibility of *An. funestus* to pyrethroids has been found to be primarily due to the increased activity of detoxification mechanisms caused by increased expression and activity of enzymes such as cytochrome P450 (P450 or CYP) and glutathione-S-transferase (GST) enzymes (Riveron *et al.*, 2014; Weedall *et al.*, 2019).

P450s (CYPs) are a large family of chemoprotective enzymes that are involved in the metabolism and detoxification of many compounds including insecticides (David *et al.*, 2013). Many African populations of adult *Anopheles* mosquitoes express elevated levels of P450 activity associated with metabolic resistance to pyrethroids and other insecticide classes. At least a dozen P450s have been linked with pyrethroid resistance, including *An. gambiae* CYP6P3 and CYP6M2 and *An. funestus* CYP6P9a and CYP6P9b, which are amongst the P450s most often found to be overexpressed in pyrethroid-resistant populations (Donnelly, Isaacs and Weetman, 2016). These resistance-linked P450s have been demonstrated to metabolise different pyrethroids and even members of different insecticide classes, suggesting the capacity to cause cross-resistance not only among the different pyrethroid chemistries, but also across a wider range of insecticides used for mosquito control (Yunta *et al.*, 2019).

This work aims to review the molecular evidence for cross- versus divergent resistance among chemicals within the pyrethroid class in relation to their structures. We focus on α-cypermethrin, deltamethrin and permethrin as the principle pyrethroids used in ITNs, thus most relevant for recommendations regarding the use of insecticide-based vector control products in areas of pyrethroid resistance. However, for comparative purposes, we include bifenthrin, etofenprox, cyfluthrin and λ-cyhalothrin, which are structurally diverse pyrethroids approved by WHO for vector control. Here, we review pyrethroid cross-resistance and its impact on the use of pyrethroids for vector control with a focus on metabolic cross-resistance associated with P450s (CYPs). However, multiple resistance mechanisms and their co-occurrence add complexity to interpretation. Relevant functional data are reviewed to identify whether key candidate genes may be more strongly associated with specific pyrethroids, and - from a molecular perspective - to assess arguments for and against using different pyrethroids as an approach to insecticide resistance management.

2.2. Methods and key findings

2.2.1. The P450 structure-activity relationship (SAR) model

We extracted functional activity data for three Type I pyrethroids (permethrin, etofenprox and bifenthrin) and three Type II pyrethroids (deltamethrin, λ-cyhalothrin and cypermethrin) that were exposed to seven recombinant P450s from *An. gambiae* (CYPs 6M2, 6P2, 6P3, 9J5, 6Z2 and 6Z3) and *An. funestus* (CYP6P9a). These enzymes are commonly associated with pyrethroid resistance (Yunta *et al.*, 2019). Values for two variables were extracted: relative insecticide binding affinity ('inhibition') of each pyrethroid by the seven enzymes; and metabolism of each pyrethroid by three enzymes that are most commonly associated with pyrethroid resistance, CYP6M2, CYP6P3 and CYP6P9a. These values were used to construct dendrograms representing the hierarchical clustering relationships among these pyrethroids in terms of these two functional activity variables. The two datasets were analysed via matrices of insecticide *vs.* P450 by Perseus v1.6.14.0 to produce two heat maps representing, respectively, clustering of relative insecticide binding affinity to each P450 and insecticide vulnerability to metabolic attack.

Pyrethroid inhibition and depletion data presented in Figure 2.1 suggests substantial variation in substrate recognition/inhibition versus selectivity for metabolism among the P450s tested. The six compounds were categorised according to their inhibition by P450s as showing potent (IC_{50} < 1 µM), moderate (IC_{50} 1–10 µM) or weak (IC_{50} > 10 µM) binding affinity (Krippendorff *et al.*, 2007), with all tested falling within the latter classes of the P450 panel (Figure 2.1A). Notably bifenthrin, which is the most structurally divergent pyrethroid molecule, has the lowest binding affinity to the enzymes panel examined (Figure 2.1A). In comparison, all pyrethroids were metabolised to some degree by these three key P450s (Figure 2B, Table 2). CYP6P3, the P450 most commonly associated with pyrethroid resistance in *An. gambiae,* demonstrated the strongest reactivity across the six pyrethroids. Specifically, CYP6P3 produced > 75% substrate depletion for all pyrethroid, and a similar pattern of is observed with its orthologue CYP6P9a, which is the enzyme most associated with metabolic resistance to pyrethroids in *An. funestus.* Metabolism data presented in Figure 2.1B showed close clustering between deltamethrin and permethrin and then etofenprox being the most vulnerable insecticides for metabolic attack by the three enzymes. α-cypermethrin shows intermediate vulnerability to metabolic attack by the three P450s between this cluster and λ-cyhalothrin and bifenthrin which demonstrated the lowest vulnerability.

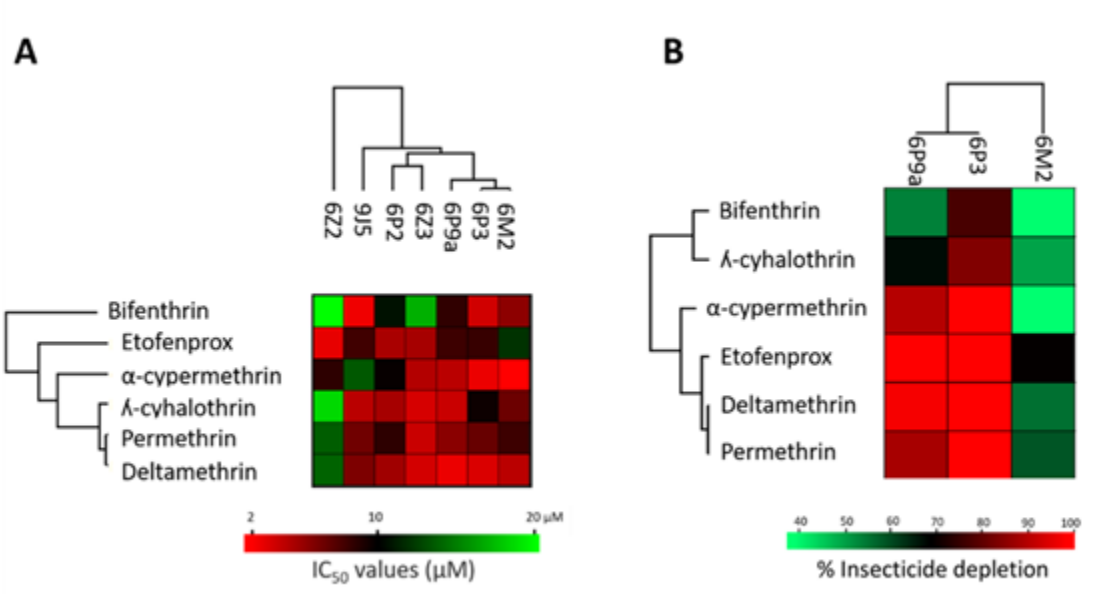


Figure 2.1. Cluster analysis of (A) Cytochrome P450s inhibition profile by the six pyrethroids and (B) pyrethroids metabolism by cytochrome P450s (data from Yunta et al., 2019).

2.2.2. Vulnerability of pyrethroids to metabolic attack in vivo

Next, we asked, is the level of *in vivo* metabolic resistance also dependent on the structure of the pyrethroid molecule? A comprehensive literature search demonstrated a scarcity of quantitative data (e.g. LD_{50}, LC_{50}) for pyrethroids in either wild or laboratory populations of malaria vectors. Most available data were related to diagnostic doses (DD) from WHO tube or CDC bottle bioassays, as discussed in Section 4.3. As an alternative approach to understanding the quantitative structure-activity relationship (QSAR) of different pyrethroids against mosquitoes, we examined the knockdown and mortality data from a previously published study (Flores *et al.*, 2013). In this study, pyrethroid susceptibility was evaluated by exposing a fully insecticide susceptible reference strain of *Ae. aegypti* (New Orleans) to multiple concentrations of permethrin, bifenthrin (Type I), and deltamethrin, λ-cyhalothrin, α-cypermethrin (Type II) in bottle bioassays. A susceptible strain is a useful model for comparing baseline metabolism of different pyrethroids within a mosquito without the result being complicated by the upregulation of metabolic enzymes which may differentially reduce mortality caused by specific pyrethroids. The knockdown data (KC_{50}) after one-hour exposure are presented in Table 2.2, and the mortality data (LC_{50}) after 24-hours are presented in Table 3. Using these data, we calculated the relative susceptibility (RS) to permethrin (Table 2.2 – 3).

Table 2.2. *Knockdown concentration (KC_{50}) of five pyrethroids against a susceptible strain of Aedes aegypti (New Orleans). Relative susceptibility (RS) to permethrin calculated by dividing KC_{50} (permethrin)/KC_{50} (the other pyrethroid).*

Insecticide	KC_{50}	KC_{50} 95% CI	x^2	RS
Permethrin	0.45	0.37 - 0.51	13.51	1
Bifenthrin	0.21	0.170 - 0.210	45.17	2
Deltamethrin	0.021	0.017 - 0.024	10.14	21
λ-Cyhalothrin	0.005	0.0002 - 0.001	15	90
α-Cypermethrin	0.0016	0.0013 - 0.0018	15.18	281

Table 2.3. *Toxicity (LC_{50}) of five pyrethroids against a susceptible strain of Aedes aegypti (New Orleans). Relative susceptibility (RS) to permethrin calculated by dividing LC_{50} (permethrin)/LC_{50} (the other pyrethroid).*

Insecticide	LC_{50}	LC_{50} - 95% CI	x^2	RS*
Permethrin	0.22	0.15 - 0.27	7.62	1
Bifenthrin	0.054	0.04 - 0.071	21.83	4
Deltamethrin	0.009	0.003 - 0.015	9.87	24
α-Cypermethrin	0.0011	0.0009 - 0.0013	43.11	200
λ-Cyhalothrin	0.0007	0.0002 - 0.001	31.02	314

Both the knockdown and mortality data indicates that, as expected, α-cyano (Type II: deltamethrin, λ-cyhalothrin, α-cypermethrin) pyrethroids are more toxic than non-cyano pyrethroids (Type I: permethrin, bifenthrin) due to high potency of Type II pyrethroids to VGSC (Narahashi, 1985; Soderlund, 2012). From the mortality data (Table 2.3,) the insecticide toxicity can be ranked (from high to low relative susceptibility) as λ-cyhalothrin > α-cypermethrin > deltamethrin> bifenthrin> permethrin, with non-overlapping confidence intervals demonstrating significant differences between all pairings. Knockdown data are broadly comparable except for a reversal of the two most potent insecticides, α-cypermethrin and λ-cyhalothrin, in terms of their 1h knockdown and 24h mortality. This relatively greater toxicity for λ-cyhalothrin over α-cypermethrin after 24h might reflect higher sensitivity of α-cypermethrin to slightly time-lagged metabolic activity (degradation and or active transportation). This observation is supported by a study carried out by Horstmann and Sonneck (2016) that suggested that the level of metabolic resistance depends on the structure of the molecule, and that structurally different compounds may still be effective as a result of detoxifying enzymes being unable to bind and metabolise them. In their study, contact bioassays were performed on susceptible *Ae. aegypti,* east African knockdown resistant (L1014S) *An. gambiae* (RSP-H strain) and metabolically resistant *An. funestus* (FUMOZ-R strain) with different pyrethroids, such as cypermethrin, ß-cyfluthrin, deltamethrin, permethrin, transfluthrin and non-fluorinated transfluthrin

(alone and in combination with the synergist piperonyl butoxide (PBO). Generally, PBO increased the efficacy of the pyrethroids containing a phenoxy benzyl moiety against the *An. funestus* FUMOZ-R strain, and against the *An. gambiae* RSP-H strain which is homozygous for the east African *kdr* mutation (L1014S). The structurally divergent transfluthrin, which contains a polyfluorobenzyl moiety, remained active against FUMOZ-R, suggesting that the P450s in this strain may have a preference to attack the phenoxy benzyl moiety. However, since PBO is also known to have adjuvant properties, this might also have been due to enhanced penetration by the pyrethroid, and it is important to link the P450 structure-activity relationship model with *in vivo* analysis to better understand the cross-resistance within a pyrethroid class.

2.3. Conclusions

- For target site resistance:
    - Some *Anopheles* bioassay studies suggest slight but significant differences in the effect of the two most common *kdr* mutations (995F and S) on permethrin *vs.* deltamethrin resistance. However, neither electrophysiological studies nor data from other taxa support these findings, although for structurally divergent pyrethroids *kdr*-effects might be more variable.
- For metabolic resistance:
  The level of a pyrethroid's vulnerability to metabolic attack (by P450 resistance markers) is an indicator of how likely metabolic resistance is to arise against the pyrethroid.
    - Permethrin and deltamethrin are the pyrethroids most vulnerable to metabolic attack by the P450 resistance markers examined.
    - Bifenthrin, ʎ-cyhalothrin and α-cypermethrin are less vulnerable.
    - Etofenprox was strongly metabolised by key P450s.
- At least in the absence of concurrent *kdr* resistance, there may be differences in how different pyrethroids perform against metabolically resistant strains: metabolic resistance may result in a more important decline in deltamethrin and permethrin toxicity than in bifenthrin, ʎ-cyhalothrin, α-cypermethrin, etofenprox and transfluthrin toxicity (based on molecular data and on *in vivo* data for transfluthrin).
- P450-structure-activity relationship (P450-SAR) models give a proxy estimate of insecticide vulnerability to metabolic attack; linking this to quantitative toxicity data in the field may help to understand the contribution of P450s to resistance.

3. Evidence from laboratory strains

N. Lissenden, G. Praulins, and R. S. Lees

This section aimed to examine data from discriminating dose assays on laboratory reared colonies to consider: a) comparability of discriminating doses across different pyrethroids, b) intrinsic variability in discriminating dose testing and c) whether evidence exists for divergent resistance in selected colonies.

Current methods for monitoring insecticide resistance are based on classifying phenotypic resistance, which is typically measured using standardised tests, such as WHO susceptibility bioassays (WHO, 2016) and CDC bottle assays (Centers for Disease Control, 2012). These tests expose mosquito populations (wild-collected females or those reared from collected larvae) to pre-defined 'discriminating doses' of insecticide and record mosquito knock down and mortality at 1 and 24-hours, respectively, post-exposure. The discriminating dose is defined by WHO (2016) as " a concentration of an insecticide that, in a standard period of exposure, is used to discriminate the proportions of susceptible and resistant phenotypes in a sample of a mosquito population". It is calculated by establishing a dose response in susceptible mosquitoes, and from the resulting data calculating either "twice the lowest concentration that gave systematically 100% mortality (i.e. LC_{100})" or "twice the $LC_{99.9}$ values" determined by this baseline susceptibility testing. In 2016, following increasing evidence that these tests may not detect changes in insecticide resistance (Toé *et al.*, 2014; Bagi *et al.*, 2015) WHO updated their guidance on insecticide resistance monitoring to include additional testing of resistant populations at 5x and 10x discriminating doses to provide further information on resistance intensity or "strength" of phenotypic resistance (WHO, 2016).

3.1. Establishing pyrethroid diagnostic doses for susceptibility testing

In 1998, a multicentre study from 7 international sites was conducted to establish discriminating doses (DD) for *Anopheles* mosquitoes against several pyrethroid insecticides (permethrin, deltamethrin, λ-cyhalothrin, etofenprox, and cyfluthrin). Known insecticide "susceptible" strains of *An. albimanus* (3 strains), *An. gambiae* (4 strains), and *An. stephensi* (3 strains) were exposed to up to 5 different concentrations of each insecticide using WHO tube bioassays. Mortality post-exposure was then analysed using probit analysis to establish a single lethal-dose for A*nopheles* for each compound, which was doubled to give the recommended DD. The DDs established remain the current recommendations: 0.15% for cyfluthrin, 0.05% for deltamethrin, 0.5% for etofenprox, 0.05 for λ-

cyhalothrin and 0.75% for permethrin. α-cypermethrin was not included in this original study; its recommended discriminating dose (0.05%) is tentative, and currently being validated by WHO in a new multicentre study (Worldwide Insecticide Resistance Network, 2019). This study will provide more robust recommendations, confirmed for individual Anopheline and Aedine species, based on data from multiple strains of each species tested in different centres, after standard methods have been validated or adapted for new chemistries. Tentative DDs calculated based on consensus data, from three centres as far as possible, are being validated in further susceptible laboratory strains. Where insufficient data can be collected, or where a methodology or DD cannot be validated, these will be excluded from the recommendations. Discriminating doses are recommended for, and routinely used in, insecticide resistance testing and monitoring globally. Under- or over-estimation of discriminating doses could therefore have an impact on the accurate monitoring and reporting of insecticide resistance in the field, and misclassification of lab-maintained strains. Similarly, differences in accuracy between insecticides could affect the comparability of bioassays. The current study re-analysed the available historical data from the WHO 1998 report to investigate the comparability of permethrin and deltamethrin DDs, the robustness of the current DDs, and how the recommended doses and the way in which they were reached align with the methods being applied in the WHO's present study for calculating DDs.

3.1.1. Methods and key findings

The evaluate the suitability of the recommended DDs, the data used to generate them were interrogated and each of the two WHO methods of calculating a DD applied in turn for deltamethrin and permethrin. These are the two pyrethroids for which there was data in the 1998 report and which are currently used in LLINs. The total number of mosquitoes assayed and the number dead were determined from the original dataset, to plot dose response curves and observe the lowest concentration at which 100% mortality was reached across the study. As results were reported as percentages, the number of dead mosquitoes was back-calculated and rounded to the nearest whole integer.

Using the first of the WHO's methods of calculating a DD, no LC_{100} could be established for permethrin, as some mosquitoes survived in one centre (Mali, *An. gambiae*, Mopti strain) at the highest concentration tested, 0.1%. The data for deltamethrin was incomplete; 0.1% killed 100% of the mosquitoes exposed to that concentration, but not all centres tested that concentration against all strains.

To establish DDs using the second of the WHO's definitions, probit analysis was used to calculate lethal concentrations and so establish DDs on three levels: 1) Individually for each study site/strain combination, 2) Pooled for each *Anopheles* species, and 3) Pooled for each insecticide overall. Site/strain datasets testing less than three concentrations of an insecticide were excluded. Probit analysis was conducted using PoloJR program within PoloSuite (Ver 2.1). The concentrations tested for both deltamethrin (0.005, 0.0125, 0.025, 0.05, 0.1%) and permethrin (0.1, 0.25, 0.5, 0.75, 1%) did not produce an even spread of mortality from 0 – 100%. The results varied between species, and between strains within species. However, for both insecticides high mortality was reached in at least one strain when exposed to the lowest concentration tested (Figures 3.1 & 3.3). These results therefore produced poorly-fitting dose-response curves, even when strains and species were pooled (Appendix 1. Figure S3.1), and in several cases, probit analysis could not calculate lethal concentration (LC) values for individual site/strains combinations, or the data were not robust enough to provide meaningful confidence intervals around the LC-values (Appendix 1. Table S3.1.). Calculated DDs are visualised in Figures 3.2 & 3.4.

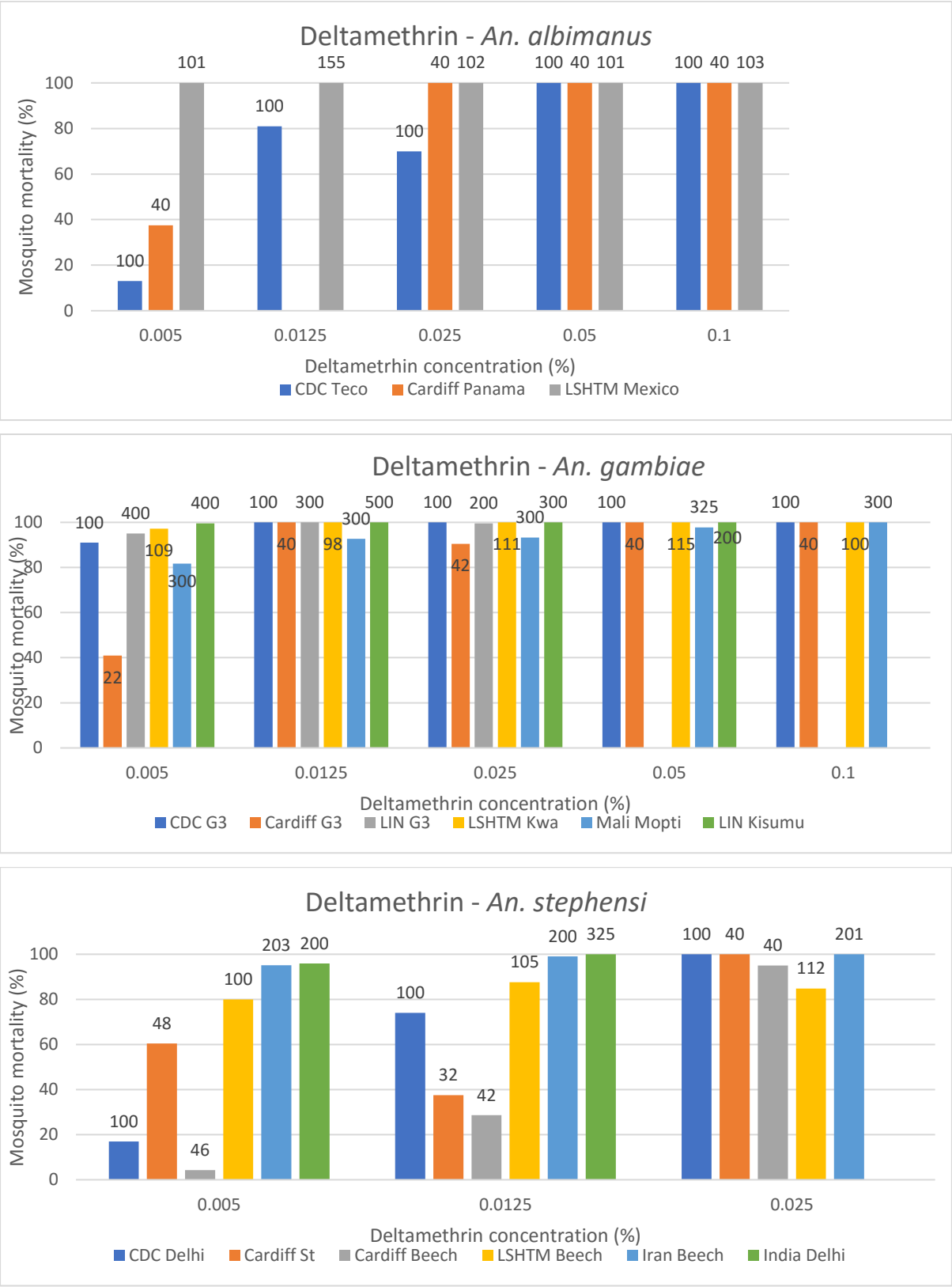


Figure 3.1. Mosquito mortality (%) following exposure to deltamethrin in WHO tube bioassays. Results are presented for site/strain combinations for *An. albimanus* (top), *An. gambiae* (middle), and *An. stephensi* (bottom). Numbers above bars show number of exposed mosquitoes.

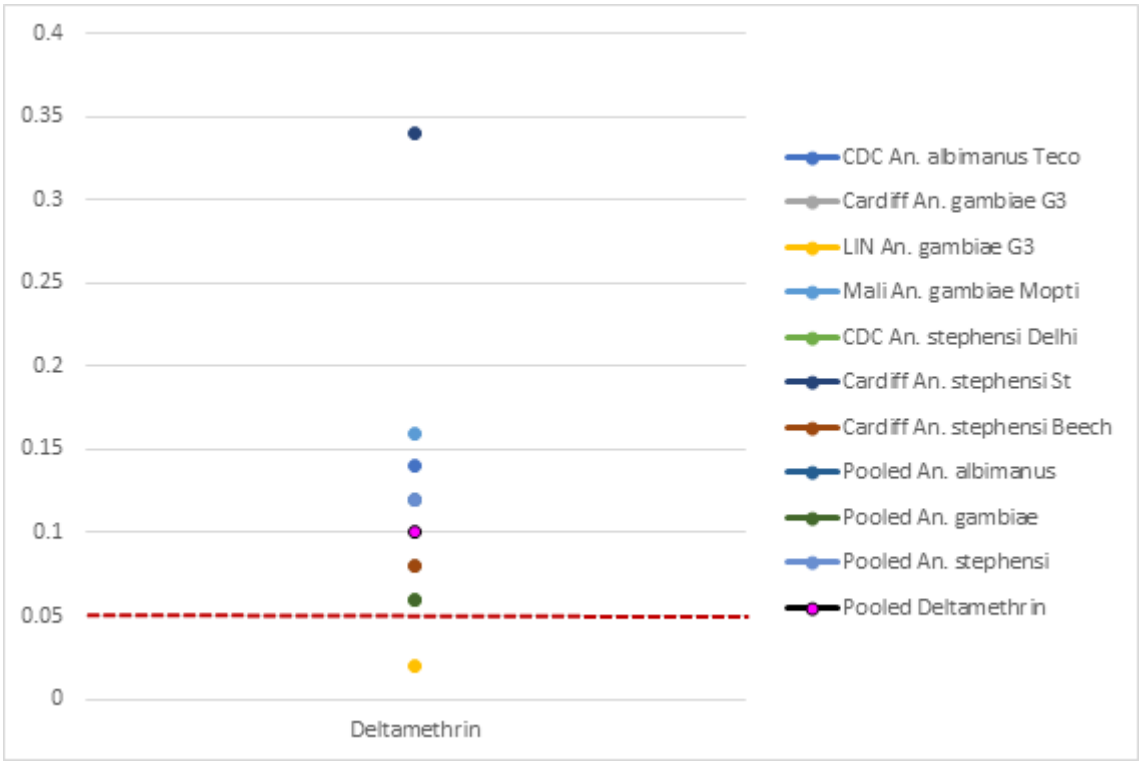
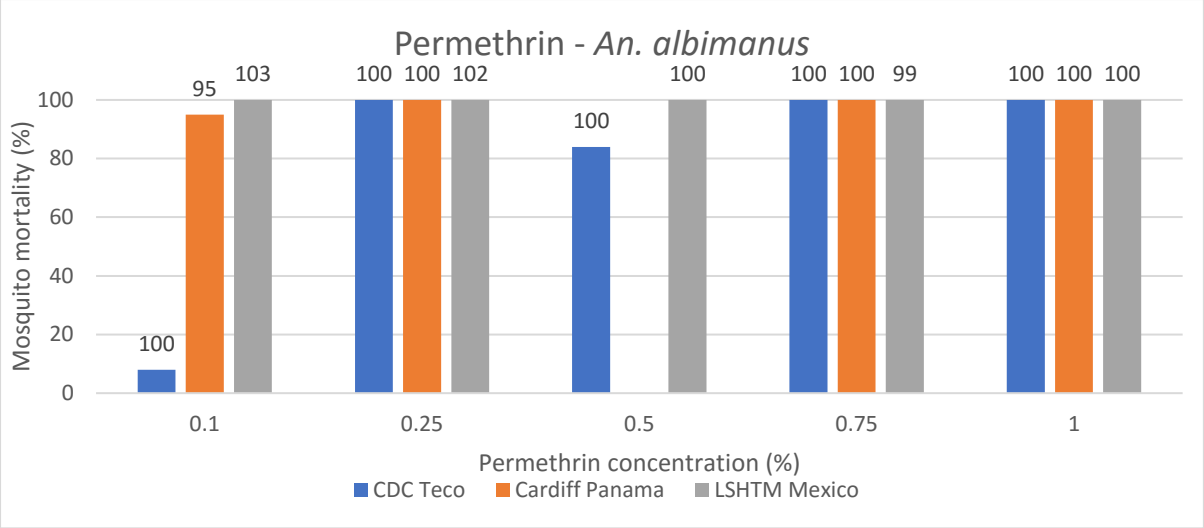
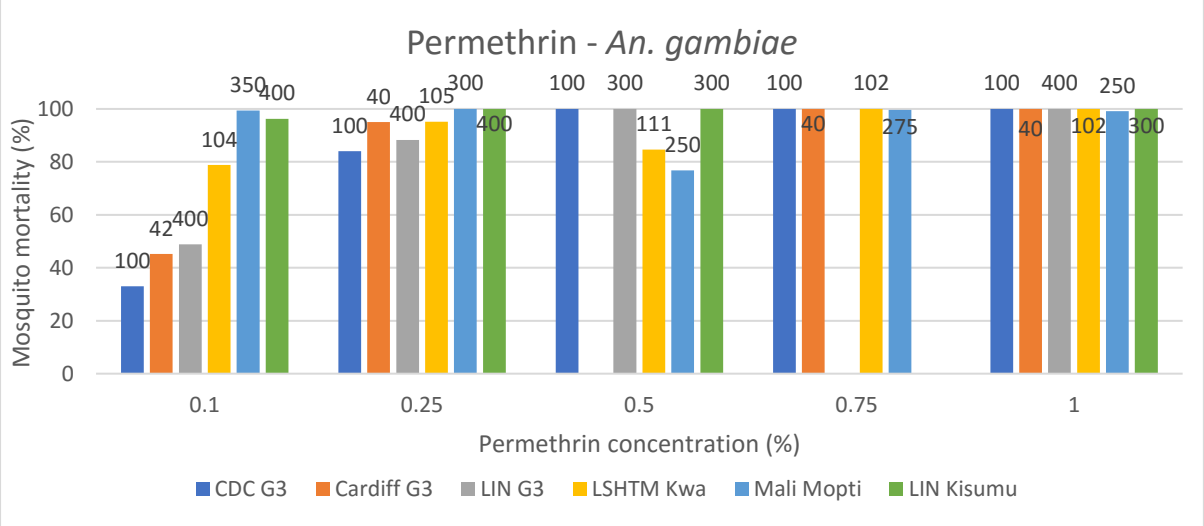


Figure 3.2. Calculated discriminating doses (%) for deltamethrin. Points show individual sites/strain combinations, and analysis pooled by species, and overall. Datasets not robust enough to calculate lethal dose matrixes are excluded. Discriminating doses are set at 2 x the calculated lethal dose at which 99% (LD_{99}) of test mosquitoes were killed. The dashed red line represents current WHO-recommended DD for deltamethrin (0.05%). One data point (LSHTM, *An. stephensi*, Beech, DD = 695.62) was omitted from the graph, to improve visualisation of other data points. A version with this included can be found in Appendix 1; Figure S3.2.





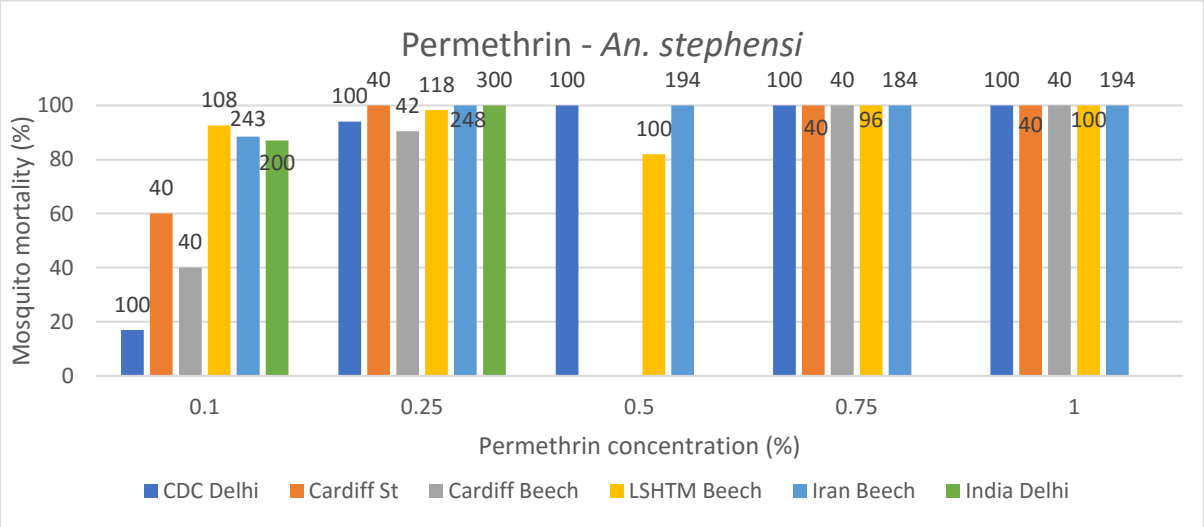


Figure 3.3. Mosquito mortality (%) following exposure to permethrin in WHO tube bioassays. Results are presented for site/strain combinations for *An. albimanus* (top), *An. gambiae* (middle), and *An. stephensi* (bottom). Numbers above bars show number of exposed mosquitoes.

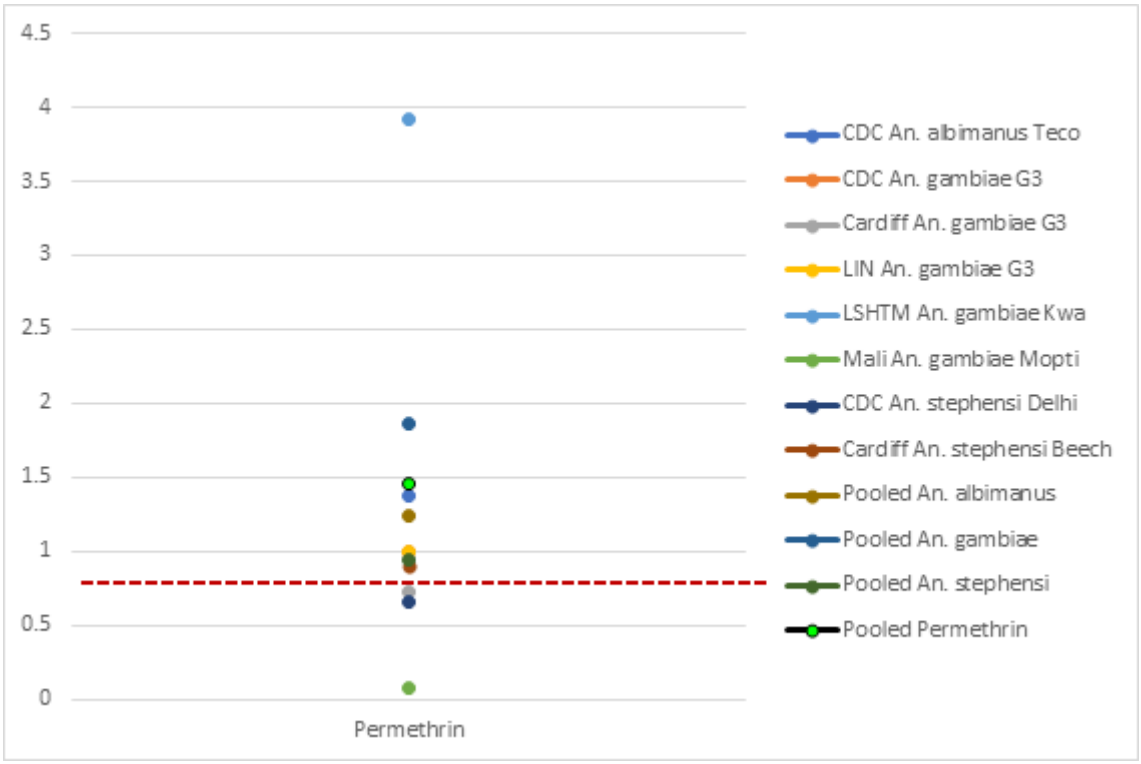


Figure 3.4. Calculated discriminating doses (%) for permethrin. Points show individual sites/strain combinations, and analysis pooled by species and overall. Datasets not robust enough to calculate lethal dose matrixes are excluded. Discriminating doses are set at 2 x the calculated lethal dose at which 99% (LD^{99}) of test mosquitoes were killed. The dashed red line represents current WHO-recommended DD for permethrin (0.75%). One data point (LSHTM, *An. stephensi,* Beech, DD = 52.32) was omitted from the graph, to improve visualisation of other data points. A version with this included can be found in Appendix 1; Figure S3.3.

In the current reanalysis, when all data were pooled, the DDs calculated were 0.1% for deltamethrin and 1.46% for permethrin (Table 3.1), ~double the final DDs recommended by the 1998 multicentre study (WHO, 1998). In the original WHO report, some summary data are provided, however it is not clear which data were included in formulating the final doses, and so it is not possible to establish why the doses calculated then differ to the ones calculated here. The report states that more weight was given to studies where mortalities were clustered around similar values. However, which studies were weighted or the methodology for weighting is not specified. Sample sizes tested per insecticide concentration were below those suggested in the WHO's original protocol. This specified 100 mosquitoes of 2-3 replicates (200 - 300 mosquitoes), however in some study sites N < 25 mosquitoes per insecticide concentration were tested. The dose-response curves seen in the current reanalysis (Appendix 1. Figure S3.1) suggests the original dose selection was inadequate. Robust dose-response relationships, which gave mortalities spread above and below 50%, were not observed. In some study sites mortality was never below 80% in the strain tested.

Table 3.1. Probit analysis of 1998 WHO multicentre study. The discriminating dose is 2 x the LD_{99}. Abbreviations: LD = Lethal dose, DD = Discriminating dose.

	Deltamethrin	Permethrin
Number exposed	8,258	9,582
LD_{99} (95% CI)	0.05 (0.023 – 1.166)	0.73 (0.452 – 2.067)
Calculated DD	0.1	1.46
Chi-square	2215.4102	2055.4866
Current WHO DD	0.05	0.75

Some factors from the 1998 study deviate from current WHO recommendations. Mosquitoes were tested at 1-to-3-day-old, whereas current guidelines state 3-to-5-day-old mosquitoes should be used. This may influence results, particularly in younger mosquitoes where their cuticle may not have hardened by day 1. There are a lack of data presented in its raw format, including mosquito mortality numbers. Information on knockdown, uncorrected mortality, temperature and humidity appears to have been collected, but is not presented.

3.2. Repeated measurement analysis

Using the established discriminating doses, resistance bioassays are routinely used to evaluate and monitor resistance in mosquito populations. When tested in well-controlled settings factors such as temperature, humidity and mosquito rearing are standardised to minimise their effect on mosquito mortality recorded from the bioassay. Looking at repeated measurements of the same populations, reared using consistent protocols, and tested under conditions whereas many variables as possible are controlled, therefore allows us to investigate what intrinsic variability stems from the assay itself.

The *Anopheles* mosquito colonies maintained by the Liverpool Insecticide Testing Establishment (LITE) and Ranson Group at the Liverpool School of Tropical Medicine (LSTM) are profiled against a suite of insecticides annually using standard WHO susceptibility testing methods. Additionally, each group selects their resistant strains every 3-5 generations with deltamethrin to ensure pyrethroid resistance is maintained (i.e. mosquitoes are exposed to deltamethrin using standardised procedures, and survivors are used to maintain the colony to sustain resistance). In some instances, this selection follows the same protocol as the WHO susceptibility testing (exposure to insecticide discriminating doses for 1-hour). When testing novel or repurposed chemistries, a positive pyrethroid control is often

used in experiments. When combined these studies represent a set of repeated bioassay (tube or bottle) measurements using the same mosquito colonies.

For each mosquito strain, available mortality data for profiling, selection or other experiments was compiled from LITE and the Ranson Group at LSTM. Only data where experimental replicates could be broken down to the tube or bottle replicate level were included in the analysis (i.e. if mortality data was only presented per experimental replicate, where tubes or bottles were pooled, this was excluded). For each strain/insecticide combination summary statistics of mortality were calculated (the range, interquartile range, mean, median, variance, and standard deviation). Box plots were created to visualise the results. A Welch's t-test was used to compare mean mosquito mortality following exposure to different pyrethroids, or to the same pyrethroid in different assays. Data were compiled in Microsoft Excel, and analysis was conducted within R statistical software version 3.6.2 (2019-12-12) (R Core Team, 2019).

3.2.1. Bioassay variability

The results from the Ranson group strains show little variability in mosquito mortality following exposure to pyrethroids in WHO tube tests (Figure 3.5). Standard deviation varies between 0.00 – 15.28 (Appendix 1. Table S3.2). However, sample replicate for these strains are generally low (<10 replicates). In LITE strains, where more sample replicates are available (>10 replicates, for deltamethrin and permethrin) greater variability is seen in mosquito mortality in tube bioassays (Figure 3.6), with standard deviations varying between 1.11 – 29.12 (Appendix 1. Table S3.2).

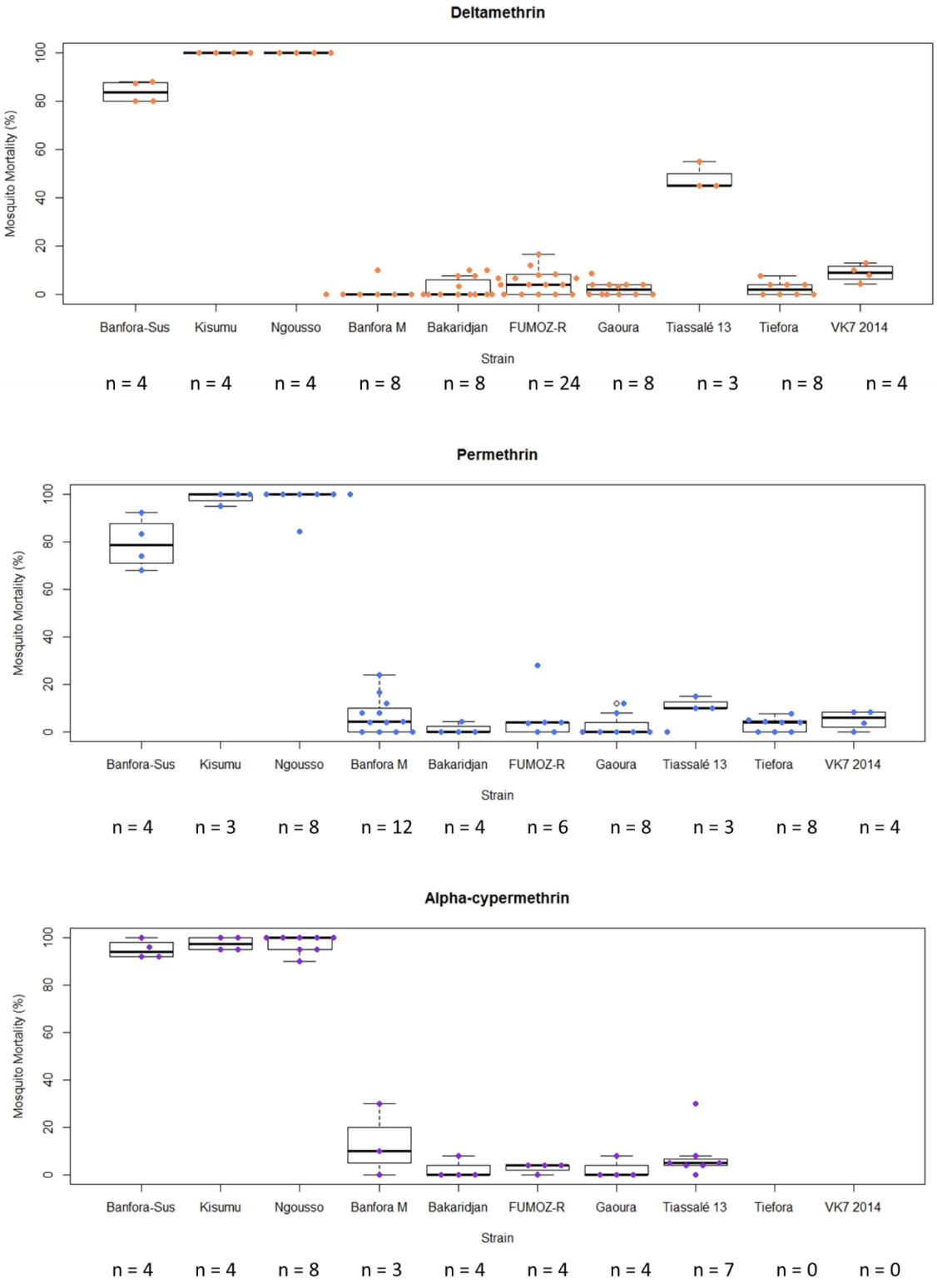


Figure 3.5. Box plot summarising mosquito mortality following exposure to deltamethrin 0.05% (top), permethrin 0.75% (middle), or α-cypermethrin 0.05% (bottom) in a standard WHO tube bioassay in Ranson group strains. Each box represents a different mosquito strain. Coloured circles and n-numbers indicate each individual tube replicate.

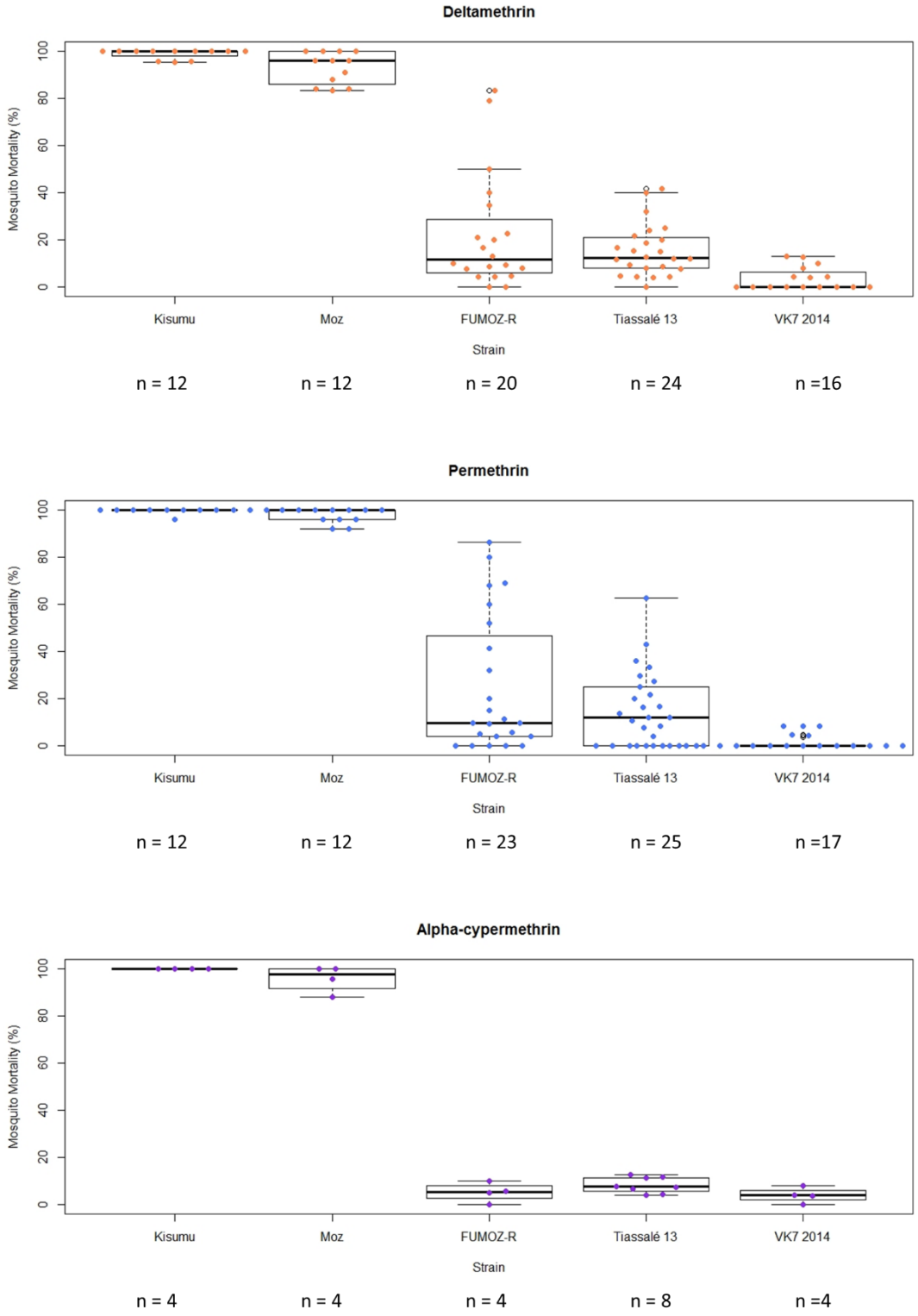


Figure 3.6. Box plot summarising mosquito mortality following exposure to deltamethrin 0.05% (top), permethrin 0.75% (middle), or α-cypermethrin 0.05% (bottom) in a standard WHO tube bioassay in LITE strains. Each box represents a different mosquito strain. Coloured circles and n-numbers indicate each individual tube replicate.

For the LITE strains, additional data were also available from mosquitoes exposed to permethrin in CDC bottle bioassays with and without simultaneous exposure to PBO in the same bottle (Figure 3.7). This is not the standard method of PBO synergism bioassays recommended by the WHO to monitor for metabolic resistance to pyrethroids in a population, and so results of the bottles with PBO in this experiment cannot be compared to field collected data described later in the report. But the data from the permethrin-only treatments give an indication of the inherent variability of the CDC bottle bioassay. The results suggest greater variability in strains showing intermediate levels of resistance compared to highly resistant or susceptible strains. This is mirrored in the PBO treatments where mortality was greatly increased in the resistant strains and the variability consequently decreased. To conclude that a lower level of variability exists in a bottle bioassay with PBO an experiment testing a range of concentrations giving a range of mortality values would be needed.

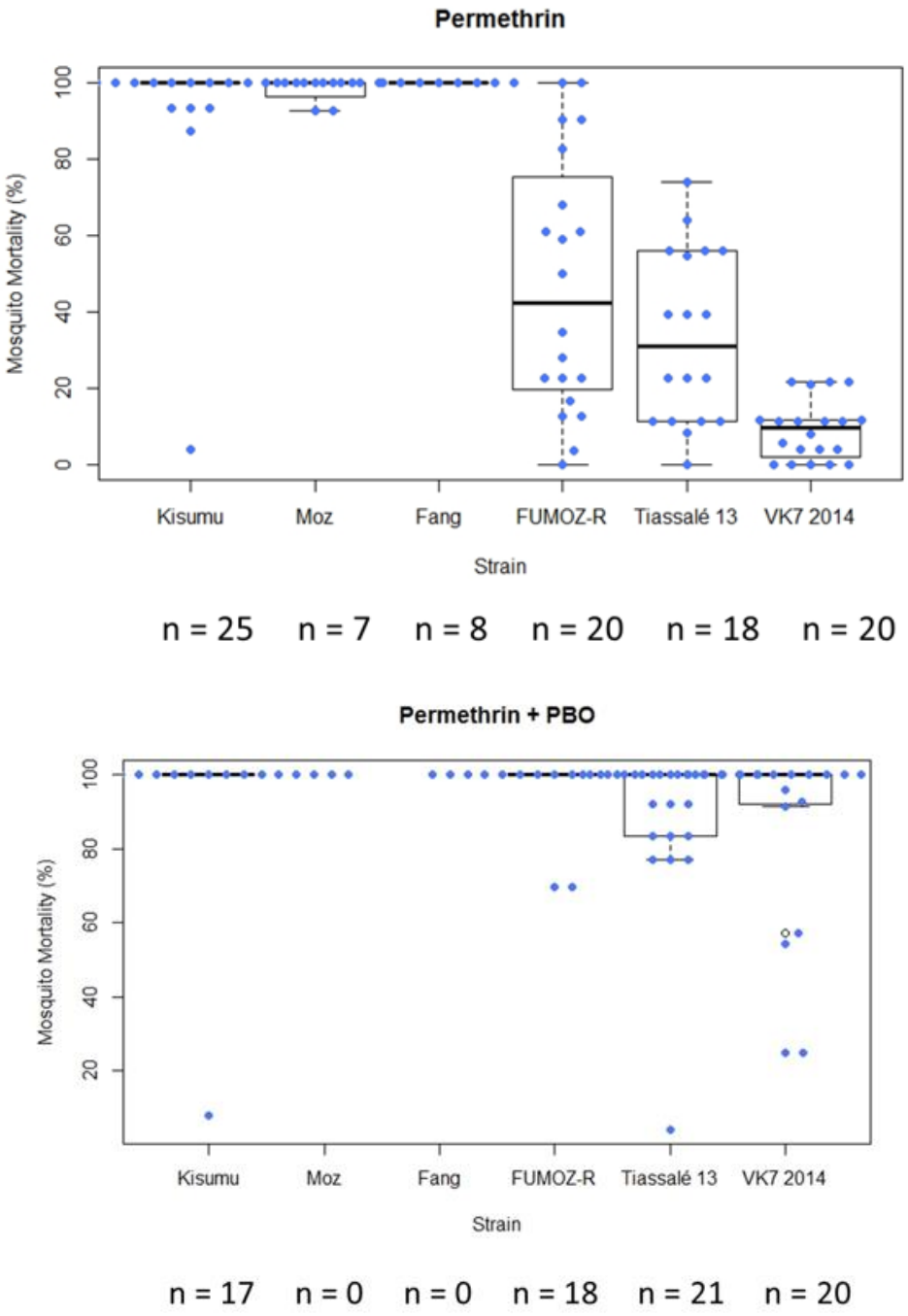


Figure 3.7. Box plot summarising mosquito mortality following exposure to permethrin 20 ug/bottle (left), or Permethrin 20 ug/bottle + PBO (right) in a standard CDC bottle bioassay. Each box represents a different mosquito strain. Strains here are those maintained by LITE at LSTM. Coloured circles and n-numbers indicate each individual bottle replicate.

Studies vary in comparability between WHO and CDC bioassay results (Owusu *et al.*, 2015), suggesting reasonable interchangeability in identifying susceptible populations, but less so when substantial resistance is present (Bagi *et al.*, 2015; Figure 3.8). Dose-response experiments are perhaps more easily performed using bottles where concentration is to be varied, but very high concentrations may

prove difficult because of issues with solubility or crystallization issues (Zhu *et al.*, 2019). If products are to be tested directly, modifications of WHO assays, rather than CDC assays are likely to be more promising. Where strains have been tested in both assays against permethrin, greater variability (higher standard deviation) is seen in the bottle bioassay (standard deviation: 3.61 - 22.50) compared to the WHO tube test. When comparing the CDC bottle bioassay with the WHO tube test (Figure 3.8) – for strains exposed to permethrin using both methodologies – resistant strains showed greater mortality in CDC bottle bioassays than WHO tube assays, but no difference was observed in susceptible strains.

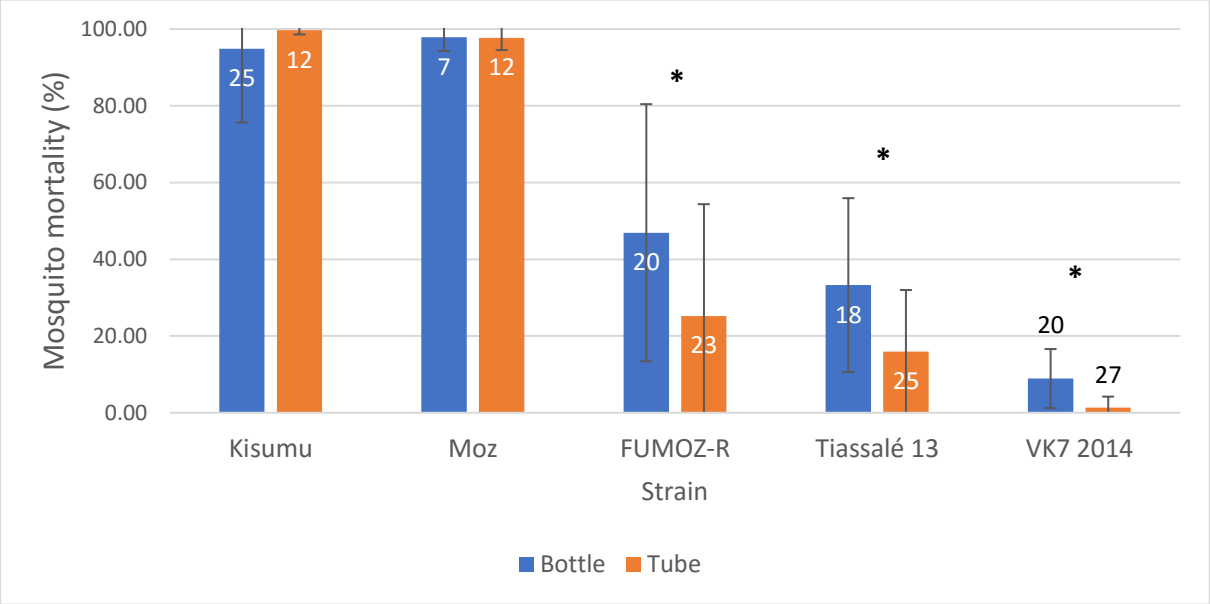


Figure 3.8. Average mosquito mortality following exposure to permethrin in CDC bottles (blue bars) or WHO tube bioassay (orange bars) in LITE strains. Numbers above or in bars indicate the number of bottle or tube replicates. Error bars show the standard deviation to indicate variability between replicates. Asterisks above bars indicate when mean mortalities are significantly different ($P < 0.05$, Welch t-test)

3.2.2. Differences in mortality among pyrethroids

Comparing mortality in the same laboratory populations tested over time against DDs of different pyrethroids allows a comparison of whether susceptibility within any of the strains differs between compounds, albeit an imperfect one given the problems with the DDs themselves and the level of variability already described. In general, intra-strain mortality following exposure to permethrin, deltamethrin and α-cypermethrin in WHO tube bioassays was similar. When strains are susceptible to a pyrethroid, or very resistant to a pyrethroid, no difference between mortality levels was seen between the pyrethroids (Figure 3.9 & 3.10). However, when resistance is in the intermediate range,

a significant difference in mortality with the different pyrethroids was observed (Figure 3.9 Banfora and Tiassalé-13 strains, Figure 3.10 FUMOZ-R and Tiassalé 13 strains). This is seen in both the Ranson group and LITE strains. When mortality in the WHO tube bioassay was plotted over time, there appears to be no obvious trends or changes in mortality from year to year (Appendix 1. Figure S3.4). All colonies are selected with deltamethrin to ensure pyrethroid resistance is maintained. In the absences of selection against all pyrethroids we could expect divergence over time if differences existed between the pyrethroids, yet there is no obvious trend towards increasing relative resistance to deltamethrin. This data was collected passively during routine monitoring of the resistance profile of colonies and a targeted investigation into the effects of selection pressure on differential resistance to different pyrethroids is needed to reach a more conclusive conclusion.

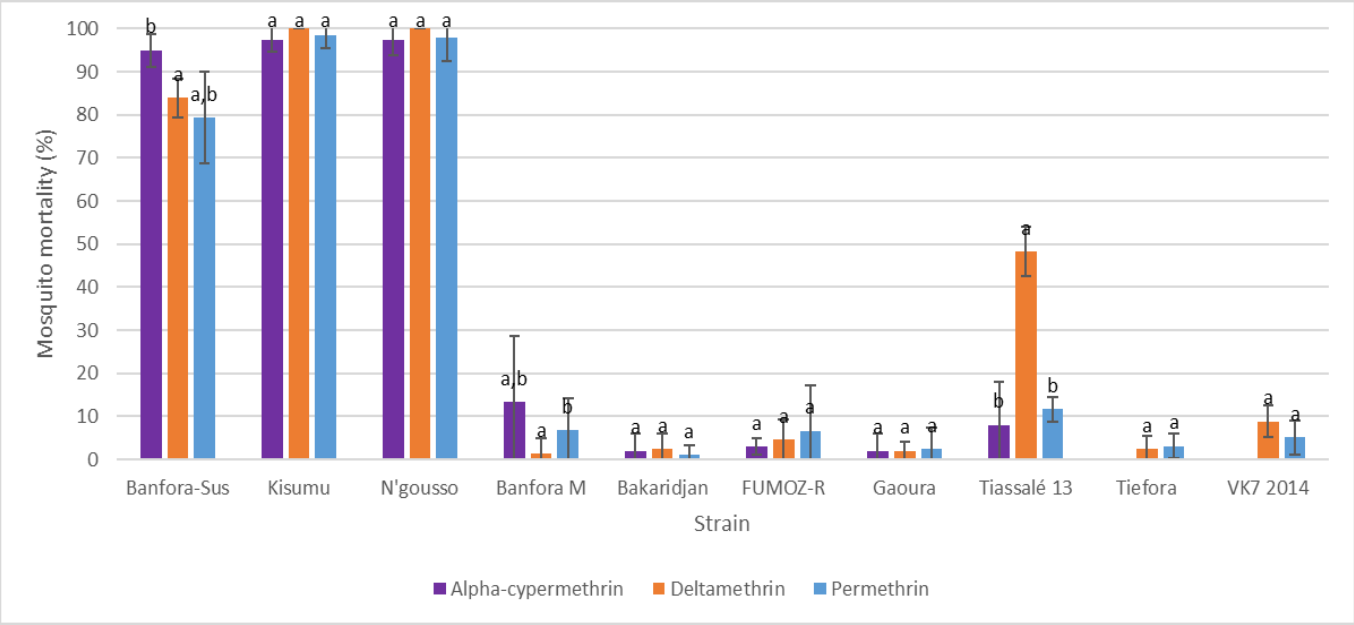


Figure 3.9. Average mosquito mortality following exposure to α-cypermethrin 0.05% (purple), deltamethrin 0.05% (orange), or permethrin 0.75% (blue) in a standard WHO tube bioassay in Ranson group strains. Bars sharing the same superscript letter are not significantly different ($P < 0.05$, Welch's t-test). Error bars show the standard deviation to indicate variability between replicates. P-values are shown in Appendix 1. Table S3.3).

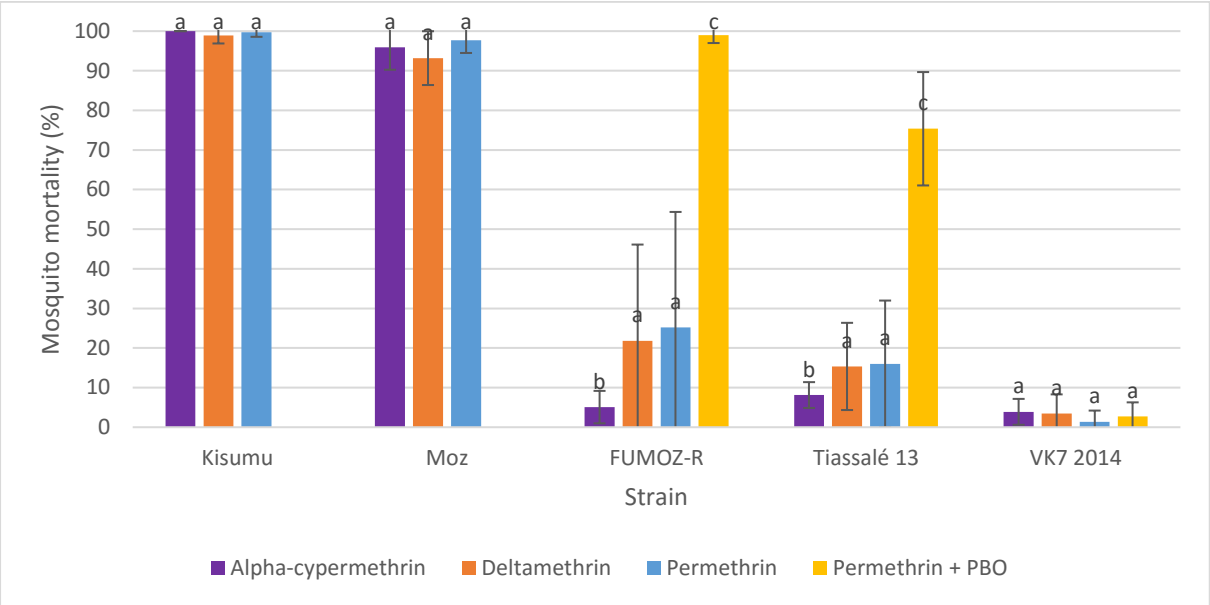


Figure 3.10. Average mosquito mortality following exposure to α-cypermethrin 0.05% (purple), deltamethrin 0.05% (orange), permethrin 0.75% (blue), or permethrin 0.75% preceded by piperonyl butoxide (PBO) (yellow) in a standard WHO tube bioassay in LITE strains. Bars sharing the same superscript letter are not significantly different ($P < 0.05$, Welch t-test). Error bars show the standard deviation to indicate variability between replicates. P-values are shown in Appendix 1. Table S3.3).

3.3. Conclusions

a) Comparability of discriminating doses across different pyrethroids.

- Publicly available data from the 1998 WHO multi-centre trial, and the associated methodology used to calculate the current discriminating doses (DD) for deltamethrin and permethrin were reviewed. Most centres within the study appear to have diverged from the agreed protocol in terms of sample size and replicates tested. The concentrations tested resulted in poor dose response curves for individual strains and the data do not appear robust. The link between the reported data and final concentrations is therefore unclear.
- Based on the publicly available data, the current DDs for deltamethrin (0.05%) and permethrin (0.75%) appear to be too low (i.e we calculated the DD for deltamethrin to be 0.1% and a DD of 1.46% for permethrin).
- Given the rationale for establishing the DDs for permethrin and deltamethrin is unclear, and the data used to calculate them was not sufficient or consistent, it is unlikely that they are comparable. This is a challenge when trying to draw reliable conclusions about relative efficacy of, or resistance to, the two pyrethroids from data collected using these concentrations.

b) Intrinsic variability in discriminating dose testing.

- In general, following exposure of characterised lab strains in WHO tube bioassays under controlled conditions the level of variability in mortality among test replicates exposed to a single compound was greater in moderately resistant strains. An experiment where PBO was added to the bottle alongside permethrin increased the level of mortality and supported the observation that greater variability is observed where mortality is intermediate. Further investigation is required to establish the inherent variability in PBO synergism assays, relative to DD bioassays.
- Variation in resistance levels within strains of the same species make it difficult to conclude if there are species differences in mortality based on this data set.
- In CDC bottle assays against permethrin, a higher level of variability in mortality data was observed in resistant strains compared to susceptible strains in this data set. When comparing resistant strains, both level of mortality and variability in mortality is greater in the CDC bottle bioassay compared to the WHO tube test in response to their respective diagnostic doses, but susceptible strain mortalities were comparable.

c) Whether evidence exists for divergent resistance in selected colonies.

- In general, following exposure of characterised lab strains in WHO tube bioassays under controlled conditions, intra-strain mortality to permethrin, deltamethrin and α-cypermethrin was similar. However, in intermediately resistant strains some divergence in mortality rates to different pyrethroids was observed.
- The laboratory strains tested in this data set have been selected with deltamethrin for up to 6 years. Despite this, trends in mortality over time do not suggest divergence between deltamethrin and the other pyrethroids.

4. Evidence from field populations

4.1. Pyrethroid resistance tests and potential sources of variability

D. Weetman

4.1.1. Insecticide testing trends in bioassays

Diagnostic dose assays (see section 3), primarily using the WHO tube bioassay in Sub-Saharan Africa, remain the most common method for resistance determination. This has led to the accumulation of large spatio-temporal datasets, which provide detailed information for investigating spatio-temporal trends (e.g. Moyes *et al.*, 2019; Hancock *et al.*, 2020). Substantial data from 2000 – 2017 are available; during this period, the resistance of over 1.07 million female *An. gambiae s.l.* and *An. funestus* were tested in WHO diagnostic dose bioassays. Testing was against insecticides from five classes, which represent three modes of action (Figure 4.1).

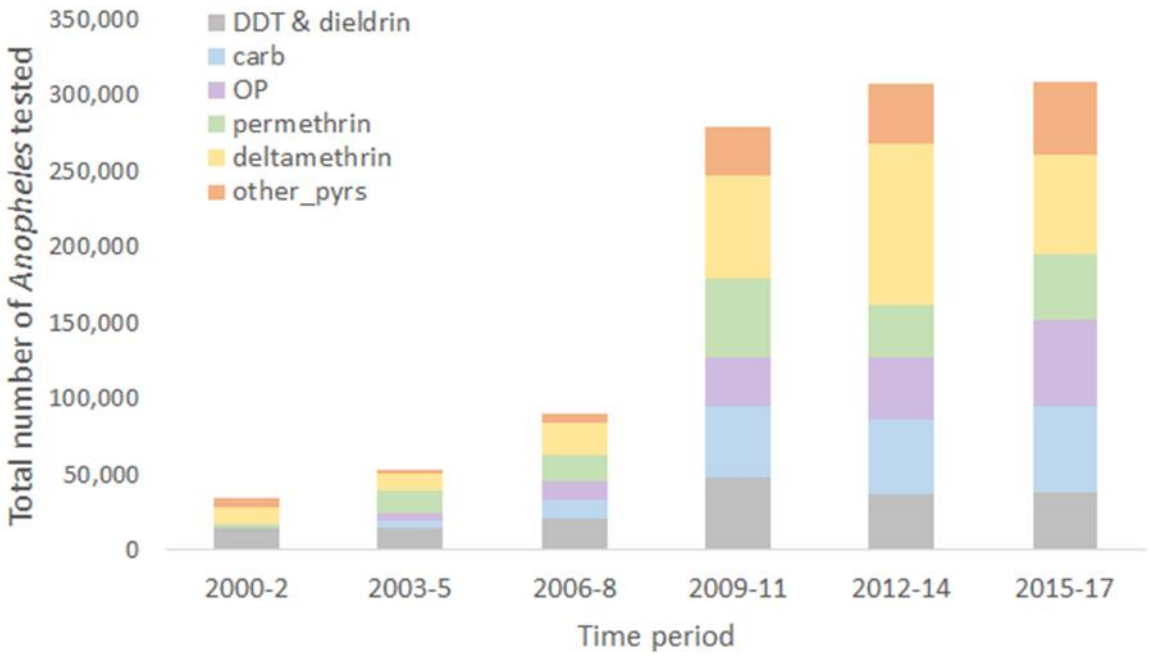


Figure 4.1. Temporal variation in the diversity of WHO diagnostic dose bioassays performed in Sub-Saharan Africa on *An. gambiae* s.l. and *An. funestus* s.l. females between 2000 and 2017. The relative proportions of insecticide types tested have changed progressively over time, with correlations between neighbouring time periods (mean r = 0.75) almost always higher than with those in subsequent periods (13/14 pairwise comparisons).

The total number of assays increased to 2008, and then rose dramatically around 2008 - 2009. The diversity of insecticides tested has also changed over time, with a relative increase in organophosphate and carbamate testing, presumably reflecting their increased use as active ingredients (AI) in IRS programmes. Conversely, a marked decrease in testing of the organochlorides, DDT and dieldrin, use of which in vector control is now largely defunct. Whilst this suggests that bioassay programmes evolve over time, they often continue to test insecticides of little or no relevance to operational decision making. For example, in the last time period shown in Figure 4.1 approximately 10% of testing capacity was expended on DDT. The rational for this continued DDT testing is unclear. Only 5% were performed on An. funestus, which might be warranted because in southern African populations DDT resistance is limited (Barnes, Irving, *et al.*, 2017; Barnes, Weedall, *et al.*, 2017). Conversely, 42% were performed in west or central African countries where DDT resistance is long-established in *An. gambiae* (e.g. Ranson and Lissenden, 2016), and where the typically less DDT-resistant species in the *An. gambiae* s.l. complex, An. arabiensis, is typically found at low frequency (Sinka *et al.*, 2016).

The frequency of pyrethroid bioassays has been relatively stable over time, constituting ≈ 50% of all tests in each time period. Testing against deltamethrin and permethrin has dominated this class. It is interesting to note that the proportion of tests on other pyrethroids (etofenprox, λ-cyhalothrin, cyfluthrin and α-cypermethrin), whilst initially quite high, fell sharply after the first time period shown and only in the final time period has approached similar proportion of around 30% (Figure 4.1).

It is questionable whether stable investment of resources in diagnostic dose bioassays on pyrethroids is warranted over a period when pyrethroid resistance has transitioned from being uncommon to near ubiquitous across Sub-Saharan Africa (Ranson and Lissenden, 2016; Hancock *et al.*, 2020). Diagnostic doses bioassays were devised primarily to document the onset of resistance, and to act as an early warning system, and not as tools to highlight higher level resistance which might challenge the efficacy of operational insecticide use. With entirely effective insecticide resistance management, free of insecticide and economical limitations and logistical constraints, the concern of operational efficacy, rather than deviation from full susceptibility, would be redundant. However, this is clearly no longer the case with pyrethroid use in vector resistance monitoring programmes. Once resistance becomes well established, diagnostic dose assays can fail to reveal underlying trends in resistance (Toe *et al.*, 2014) and are prone to bias and variability in results, leading to potentially severe quantitative errors. Moreover, as a tool to facilitate comparison between resistance to different insecticides, diagnostic

doses must be equivalent, a requirement which evidence reviewed in Section 3 does not seem to support.

For any bioassay results, despite known issues with repeatability and susceptibility to sources of variation (e.g. temperature, humidity, age of mosquito – reviewed below), data from replicate tubes are seldom reported. Typically, variability in diagnostic dose bioassay results is represented as binomial confidence intervals and/or contingency tests if comparing treatments, but these ignore *intra*-test variability, which can be substantial (see Section 3). When assessing differences – across time, space, or between insecticides it is important to account for this intra-test variability using improved methods of analysis. For example, a binomial-link general linearised model (GML) with each individual test (e.g. results from each tube or bottle) as the unit of data entered. This analytical suggestion applies to either to discriminating dose assay data or when applying the more quantitative methodologies described below.

Overall, and linking also with results from Section 3, the above suggests that resistance assessment programmes need to be more flexible in order to better meet operational needs, in terms of prioritising the insecticides to be tested, and also in basic data reporting and analysis methodologies to allow incorporation of innate intra-test variability.

4.1.2. Quantitative comparative assessment of resistance levels

Whilst diagnostic dose bioassays fully retain their utility when resistance is absent or uncommon, better alternatives are required when resistance is established. Available evidence and mechanistic theory suggest that this will be the case for most malaria vector communities for all or most pyrethroids across Sub-Saharan Africa. Given their current irreplaceability, monitoring of pyrethroid resistance levels (as distinct from qualitative presence/absence of resistance) remains a high priority. In the absence of simple, highly diagnostic DNA markers for operationally-significant pyrethroid resistance, which are becoming available for *An. funestus* (Mugenzi *et al.*, 2019; Weedall *et al.*, 2019), but remain under development for *An. gambiae* (Donnelly, Isaacs and Weetman, 2016; Eric R Lucas *et al.*, 2019; Harun Ngorje, unpublished data) this challenge may be addressed using fully- or semi-quantitative phenotypic bioassay methodologies.

4.1.3. Dose-response bioassays

Dose-response bioassays are a traditional methodology providing quantitative assessment of resistance and are appropriate to estimate the level and variability of response to insecticide exposure displayed by a population, or among populations. In such protocols, the combination of a specific concentration x time represents a measure of insecticide dose. Thus, mosquitoes are exposed to a range of insecticide concentrations for a fixed time period, or a fixed insecticide concentration for a range of exposure times. The latter approach is less common and can be problematic if using a standard diagnostic dose when resistance is well established, because required exposure times to kill a high proportion of mosquitoes become unfeasible (Mawejje *et al.*, 2013). From the regression line fitted to data, typically using probit or logistic regression, the doses killing specified percentages of the test cohort (i.e. the doses lethal to a given percentage) can be estimated, most commonly at the 50% and then one or more of the 90%, 95%, and 99% lethal doses (LD_{50}, etc., also equivalently shown as LC_{50}, etc.). Significant differences in the slopes and intercepts of the regression line can indicate variation in population profiles for responses to insecticide. Whilst the dose-response methodology can indicate variation in resistance among populations, quantification of resistance level per se requires comparison with a susceptible strain at a specific killing dose point (most commonly the LD50) to estimate a resistance ratio (RR), which can be compared between insecticides. However, even without this comparator, insecticides could be compared by estimating lethality at a dose for each insecticide representing the recommended dose of insecticide (or a fraction or multiple thereof, depending on population resistance level) in formulations used for, or on/within interventions.

A significant drawback of the dose-response methodology is that testing ≥ 50 females at typically 6-7 dose points per insecticide requires very large collections of mosquitoes for testing, with a realistic minimum requirement of at least fourfold the numbers required for diagnostic dose assays. This is likely to both reduce the number of insecticides tested and also to extend the testing period required over multiple days. If the overall testing cohort is reliant on multiple sub-collection sites, with concomitant potential to vary in mosquito composition, it is important that replicates across treatments (either different concentrations or different insecticides) should be balanced to prevent sampling variance becoming treatment-bias.

A very seldom-applied (no cases documented in IRMapper to date, Knox *et al.*, 2014)) variant on the dose-response methodology could be to test products directly using exposure time as a variable. Whilst potentially more directly operationally relevant, WHO tube and cone test protocols will need

to be modified for this purpose to allow cessation of exposure at precise times. Yet, if it is valid to equate mosquito knockdown within an assay as a proxy for mortality, indefinite-duration cone test assays could be applied, in which proportionate mosquito knockdown is recorded at intervals or on an individual basis if video recording and analysis is employed (see Section 5).

4.1.4. 'Resistance intensity' bioassays.

Where reliable diagnostic doses are available, a compromise strategy, increasingly adopted for pyrethroids in particular, is to apply a stepwise assessment procedure (WHO, 2016), whereby if resistance is detected at the diagnostic dose (denoted x 1) progressively higher doses of the insecticide are tested on the mosquito cohort until resistance is no longer detected (e.g. x 5, x 10). This can yield key parts of the information provided by dose-response assays and will typically require fewer test individuals. Whilst this methodology is practical, as a tool for comparison between insecticides it remains predicated on the relative accuracy of the original diagnostic doses for each insecticide, which appears problematic (Section 3). An alternative could be to apply insecticides at fractions or multiples of operationally-determined doses as suggested above, but this loses the current ease-of-application of having pre-available WHO treated papers, and also would become complicated by availability of multiple competitor products using the same active ingredient at different doses.

Although demanding in terms of mosquito requirements for testing, dose-response bioassays retain utility as the preferred method for comparative assessment of insecticide resistance among insecticides. Ideally, and to facilitate comparisons across studies, they should also involve a standard fully susceptible laboratory strain for calculation of resistance ratios, however, where not available they should at least be capable of providing meaningful comparative assessments among insecticides within a single study. Depending on the objective and range of competitor products of interest, a dose-response methodology testing insecticidal products directly may be worth consideration.

4.1.5. Sources of variation and error in bioassays

4.1.5.1. Environmental and mosquito conditions

WHO (2016) give precise parameter values for some of the key environmental conditions that should be adhered to when carrying out bioassays. Whilst poor larval rearing conditions of crowding and/or low food can have extreme effects on bioassay results (Owusu, Chitnis and Müller, 2017) such

conditions are typically relatively easily controlled by bioassay practitioners. Time of day effects do not seem to have been explored in the literature but circadian rhythmicity of many detoxification genes suggest that mosquitoes tested at night may not show the same patterns as those tested during the day (Rund *et al.*, 2011; Balmert *et al.*, 2014). This may be significant for operational interpretation of results, considering that African *Anopheles* vectors typically bite at night, but this is unlikely to be a source of variability affecting bioassay data, because all tests are performed during daytime hours. Nevertheless, reporting of testing times along with bioassay results would be good practice to be adopted more widely.

In contrast, under field conditions WHO-specified temperature and relative humidity conditions are often difficult to achieve and maintain, and the effects of variation can be highly significant. As part of a genome- wide association study with sampling and testing conducted in a field insectary in Uganda, Weetman *et al.* (2018) detected a strong decline in *An. gambiae* mortality as humidity increased (Figure 4.2). In this study, temperature also varied but did not independently account for significant statistical variation in mortality. In the WHO-IIR (impacts of insecticide resistance multi-centre trial, temporal repeatability of results from sentinel sites in Sudan was poor (Implications of Insecticide Resistance Consortium, 2018), a significant contributory factor appears to have been variability in temperature and relative humidity, which correlated strongly (Figure 4.2). Respectively, these decreased and increased diagnostic dose pyrethroid bioassay mortality in the *An. arabiensis* tested. Interestingly, this is the opposite directionality to that observed in Ugandan *An. gambiae* and may reflect the differences in aridity tolerance between the species (Coetzee, Craig and le Sueur, 2000). Significant, but inconsistent effects of temperature on bioassay mortality are also reported among laboratory colonies of *An. stephensi* (Glunt *et al.*, 2014), *An. arabiensis* and *An. funestus* (Glunt *et al.*, 2018). Whether or not the contrast in humidity effects between studies reflects the different physiological adaptations of the species studied, such variability highlights the difficulty in predicting and statistically controlling for temperature and humidity effects, which are likely to depend quantitatively on the humidity-temperature optimum-tolerance profile of the population tested. Nevertheless, it is essential that studies record and report these variables accurately, so that caveats can be applied to comparative conclusions or datasets obtained under extreme conditions may be excluded in subsequent meta-analyses.

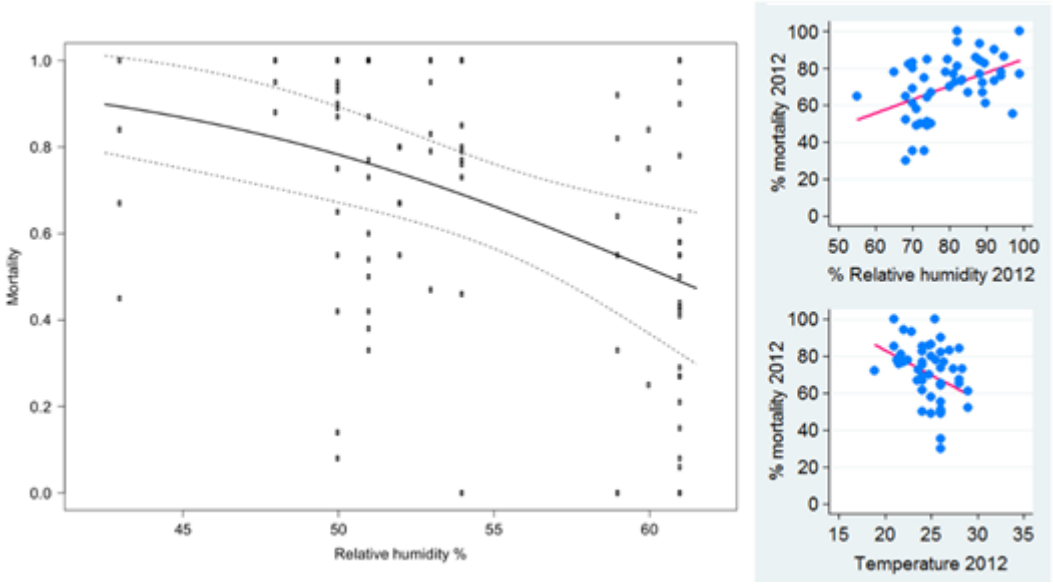


Figure 4.2. Effects of environmental conditions on bioassay mortality in field insectaries (A) humidity impacts on WHO tube diagnostic dose (DD) permethrin bioassays performed on Ugandan *An. gambiae* (Weetman *et al.*, 2018); (B) humidity and temperature impacts on WHO deltamethrin DD bioassays of Sudanese An. arabiensis (Implications of Insecticide Resistance Consortium, 2018). All regression lines are highly significant.

Age of mosquitoes tested is an important consideration, and multiple studies have shown that insecticidal mortality in pyrethroid bioassays performed on *An. gambiae*, *An. coluzzii* and *An. arabiensis* increases with mosquito age (Glunt, Thomas and Read, 2011; Chouaibou *et al.*, 2012; Kulma, Saddler and Koella, 2013; Mbepera *et al.*, 2017; Machani *et al.*, 2019) with similar results shown when exposed to standard pyrethroid ITNs, or the carbamate insecticide bendiocarb (Christopher M. Jones *et al.*, 2012), metabolic resistance to which may share commonalities with pyrethroids (Edi *et al.*, 2014). Whether this pattern is universally true across insecticides and resistance mechanisms is unclear. Recent work on pirimiphos methyl resistant *An. gambiae* from Ghana, in which resistance is strongly determined by combinations of target site mutations, shows no trend in mortality over ages spanning 3-15 days (Essandoh, 2020). However, provided mosquitoes are reared in the laboratory from larvae or eggs, results from bioassays using either deltamethrin or permethrin appear to ubiquitously show significant age-dependent mortality increases, which argues for the current approach of targeting young adults (>1-day-old), i.e. the least susceptible age group, for insecticide testing.

Physiological condition of females, not directly related to age, may play a less predictable role in bioassay mortality variation. The effect of blood-feeding has been primarily studied in laboratory strains of *An. arabiensis* or *An. funestus* in which a moderate, and transient, reduction in permethrin and deltamethrin mortality after a single blood meal has been detected (Spillings *et al.*, 2008; Oliver

and Brooke, 2014), which has recently been replicated in field-collected samples of *An. gambiae* from Kenya (Machani *et al.*, 2019). The proposed mechanism for this is upregulation of a vast number of detoxification genes, in response to the oxidative stress caused by intake of blood by female mosquitoes (Marinotti *et al.*, 2006). The magnitude of effect appears to be dramatically greater if multiple blood meals are taken in the females' life before insecticide exposure (up to 60% reduction in mortality for permethrin and deltamethrin, even in 21-day-old females) (Oliver and Brooke, 2014). Further studies on the same strains showed that multiple bloodmeals appear linked to a sustained enhancement in the ability to defend against oxidative stress, a common toxic effect of pyrethroid exposure (Oliver and Brooke, 2016). Repeated sub-lethal prior insecticide exposures might have a similar effect, but results to date are inconclusive (Glunt, Thomas and Read, 2011), possibly because of the conflicting effects of priming via enzyme induction from insecticide pre-exposure, and delayed effects of sublethal exposures on mortality (Viana *et al.*, 2016). In the absence of additional studies, the ubiquity and magnitude of the effects of repeated sub-lethal insecticide exposure, and more concerningly, repeated blood-feeding are difficult to predict, but suggest, in combination with the more estimable age-effects, that performing bioassay testing on adult females caught directly from the wild may provide very variable or even biased results.

4.1.5.2. Mosquito sampling

A common feature of most published works describing bioassay data is relatively poor details of sampling, which is usually performed following an opportunistic plan. Generally few details are provided to describe the range of site types, and often only a single GPS location is given, which probably can be assumed to represent an approximate central point for sites contained within a polygon of unknown size (Moyes *et al.*, 2019). For comparative studies involving bioassay data this is problematic because (a) chances of repeatability are lowered by lack of collection detail, and (b) samples may lack independence as biological replicates, which may introduce bias or inflate statistical power (compounding further the issues from pooling replicate assays noted earlier). *A priori* the predicted magnitude of this effect is expected to depend on the collection method employed. If adults are collected, they may be either tested directly (noting the inherent problems with testing adults with unknown variation in physiological status and age described above) or used to obtain eggs, which may be combined and reared for adult bioassays. The adults would typically be assumed not to be closely related; whilst if their eggs are used, the level of relatedness in the resultant sample would be expected to be roughly proportional to the number of families combined. However, for the *An. gambiae* complex the most common method of obtaining samples involves collecting larvae from

breeding sites, presenting a potentially significant, but unknown, likelihood of sampling many siblings. A strategy of collecting from as many local breeding sites as possible might reasonably be expected to ameliorate this problem to some extent. However, to our knowledge, and despite the testing of well over a million *Anopheles* malaria vectors in Sub-Saharan Africa using WHO assays, populated with samples obtained using these collection methods, there has been no study examining relatedness levels in collections made following any of the above strategies. As part of genome wide association studies using a bioassay-based insecticide resistance phenotype, we collected larval samples for bioassays in Yaoundé, Cameroon and Dodowa, Ghana in 2006 (Weetman *et al.*, 2010) and adults from Tororo, Uganda in 2008, from which we obtained offspring for testing (Weetman *et al.*, 2018). Further samples were obtained from recently- and long-established colonies at LSTM, and all samples were genotyped using a custom Illumina array. More recent collections were made from over 50 locations (each represented by several breeding sites within a radius of a maximum of a few kilometres, and often much less) across southern Ghana in 2016. Genomes of a random sample from each collection were sequenced at low coverage (Essandoh, 2020). In each dataset, relatedness categories among the samples were estimated (Figure 4.3).

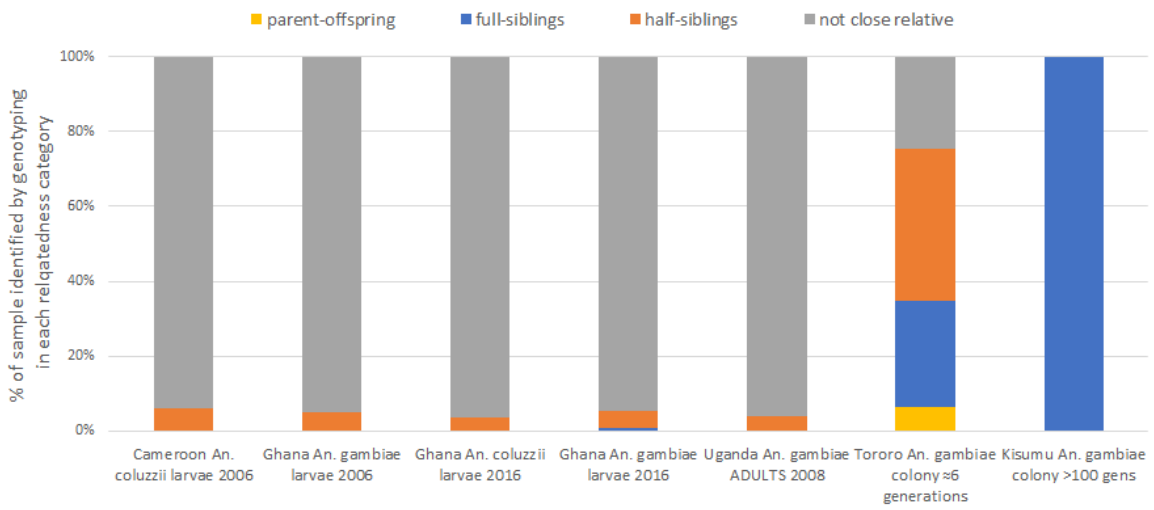


Figure 4.3. Genotype-based identification of close relatives in female samples collected as larvae from nearby collection locations in 2006 and 2016, and as adults in 2008; samples from a recently established, and a very long term colony are also shown for comparison (Weetman, Essandoh unpublished). Results from 2016 are estimated from data on ≈160 samples for each species at 2229 chromosome 3 SNP markers. All other data are from 286 chromosome 3 SNP markers (Weetman et al,, 2010) with field sample sizes of ≈180 for adults and 600-700 for larvae.

Results proved to be surprising. Larval collections in 2006 contained only approximately 5% of siblings, and those from within the same locations (i.e. sets of local breeding sites) in the 2016 collections

showed a similar overall average, though occasional sites showed much higher values (maximum = 46% related as half- or full siblings). This suggests that relatedness within breeding sites is much lower than might typically be assumed, and samples dominated by siblings is probably the exception, rather than the norm, provided efforts are made to sample as many locally-accessible sites as possible. This is concordant with recent results from *An. arabiensis* showing that productive breeding sites contain many larvae because they contain many families, rather than large numbers from single or few families (Odero *et al.*, 2019). Relatedness among the adults collected from houses in Uganda is similar to that among the larval collections, though all the estimates contrast very markedly with the majority of close relatives seen in the recently established colony, and the ubiquity of close kin in the Kisumu lab strain (Figure 4.3). Overall, these results suggest that with reasonable diligence (supported by provision of more sampling details in publications), most larval samples of *An. gambiae* might be assumed unrelated in locations where multiple breeding sites are available, providing little problem with the assumptions of independence for statistical models. Where obtaining larvae is difficult, obtaining eggs from many females could present a reasonable alternative, and either may yield relatively large collections for testing.

A final consideration in sampling is species identification. Failure to differentiate morphologically-cryptic species within complexes or groups can create significant biases when comparing results among studies. Where relative species composition varies in space or time, failure to identify which are being tested can lead to misinterpretation of causality if insecticide resistance differs, which is often the case for *An. gambiae* or *An. coluzzii* vs. other species complex members (e.g. Opondo *et al.*, 2016). Multiple cheap and reliable molecular assays available to identify species and their application is crucial, though initial morphological identification to the level of species complex or group is advisable (Erlank, Koekemoer and Coetzee, 2018).

4.1.6. Conclusions

- Trends in diagnostic dose bioassay data from 2002-2017 show that the insecticides tested have changed over time but testing of either defunct insecticides or those with very well-established resistance persists. Diagnostic dose assays were designed to identify the emergence of resistance and are poor tools for quantitative analysis of resistance levels where resistance is established. More consideration of the purpose of the testing and operational significance is required to use the available resources to most effectively monitor for resistance to current products and inform deployment decisions.

- Resistance intensity assays provide improved resolution of resistance level but for comparisons among insecticides, suffer from the same problem as the diagnostic doses on which they depend – an apparent lack of parity across insecticides as described above (*Evidence from laboratory strains (a)* point 3). The most comparable methods for comparing different insecticides are dose-response assays (which are not dependent on existence or accuracy of diagnostic doses) and vary either insecticide concentration or exposure time. However, large numbers of mosquitoes are required and an increase in direct testing of insecticidal products as part of monitoring programmes may provide more efficient operationally relevant information to aid decision making.
- With more insecticides or products to test, larger numbers of mosquitoes are required, whilst utilising collection methods that yield representative population samples. Larval collections often provide the best option for intensive sampling, but might yield many closely related individuals, biasing results. However, we provide results that show that with pragmatic, but carefully performed sampling average relatedness is low, supporting the statistical validity of large larval collections.
- All bioassays are vulnerable to very strong effects of humidity and temperature, in addition to other environmental effects more easily standardized by the user. Indeed, data show that moderate changes in conditions can affect mortality enough to change a classification of a cohort of mosquitoes from susceptible to resistant or *vice versa*. In addition to avoiding testing in uncontrolled conditions where possible, improved reporting of sampling, rearing and testing conditions is crucial to allow consideration of possible biases when interpreting and using data.

4.2. Analysis of insecticide divergence and data noise in the field

M. Kont, B. Lambert, T. Churcher, C. L. Moyes, P. A. Hancock

4.2.1. Background

Data from discriminating dose and resistance intensity bioassays on field populations are examined to look for evidence for existence of divergent resistance between pyrethroids. Each bioassay type (discriminating dose or resistance intensity) are examined separately to gain insight from their respective greater breadth and precision. These data are also compared to results from a systematic review of experimental hut trials comparing different Type I and Type II pyrethroid insecticidal nets (dipped nets and ITNs). Experimental hut trials are the most widely used entomological method of evaluating ITNs in field conditions. They are not a resistance monitoring tool as they require specialist

infrastructure, personnel and typically take several months to complete. Nevertheless, they provide the current best entomological characterisation of how resistance influences the efficacy of ITNs and therefore provide a link between the level of resistance and the impact that resistance can have on public health.

4.2.2. Rationale and methods

All tests are prone to measurement error and Sections 3 and 4.1 highlighted different biological reasons why assay results may vary. Measurement error will be greatest in the discriminating dose bioassay as each point represents a single replicate. This measurement error may be reduced in resistance intensity bioassays due to each study combining data from ideally 6 sets of repetitions (i.e. a set for each dose examined). Experimental hut trials have the greatest number of repetitions (typically collecting data from every night over at least 36 nights) though the greater realism of the assay means there are potentially more sources of variability than in the discriminating dose and resistance intensity assays which are conducted in more controlled conditions.

There are insufficient bioassay data available that directly compare Type I and Type II pyrethroids in the same experiment. To overcome this problem, spatio-temporal analyses are used to group mosquitoes together in space and time to increase the number of comparisons that can be made. The spatial scale to use for these analyses is unclear as the distances over which mosquito populations vary in the wild is generally unknown and is likely to vary considerably. There is similar uncertainty about how the level of resistance in the vector population changes over time as the dominant malaria vector is known to vary throughout the transmission season within and between species complexes. In practice the optimal scales to use will depend on the availability of data and the question under investigation. Though it would be interesting to understand the fine scale variability within and between mosquito species, here we are primarily interested in how differences in mosquito mortality influences the decision on which pyrethroid ITN to deploy within a particular region. For practical reasons, these decisions cannot be made at fine geographical scales, such as at the level of the village, so the analysis must be conducted at the spatial scale at which ITNs are deployed. This varies among countries but is typically high, with the same ITNs being deployed over 100s of kilometres. Similarly, ITN procurement for mass distribution programmes occurs every three years so small-scale temporal changes are less important than overall trends over multiple transmission seasons.

Unfortunately, most bioassay results currently available do not differentiate between species, and, those that do, report the dominant vector only to the species complex level (Moyes *et al.*, 2019). We also do not know how the vector species changes over time in the regions considered in the analysis so it is therefore necessary to omit mosquito species as a covariate from the majority of these analyses. This assumption, though unfortunate, means that we assume that the mosquitoes sampled in an area are representative of the local vector population in that region over the year. Despite this, differences between species should not be discounted or considered unimportant, as highlighted by the example summarised in Section 4c which does differentiate between different species complexes. Analysis is restricted to the African continent due to the availability of data.

4.2.3. Discriminating dose bioassay

There is clear geostatistical evidence of a strong association between the level of mortality induced by Type I and Type II pyrethroids (Figure 4.1A-C). This 2018 study tested associations among resistance to deltamethrin, λ-cyhalothrin and permethrin, and to DDT (Hancock *et al.*, 2018). This study used susceptibility test results and a geostatistical model that distinguished the spatial and temporal trends in the susceptibility test data from noise in these data. This version of the model did not use covariate data. Two sets of models were run independently; one in west Africa and one in east Africa, reflecting the two regions with sufficient data.

We found strong associations among spatio-temporal patterns of resistance to all three pyrethroids tested. A linear model of coregionalization (LMC) that allowed interactions between resistances across all three pyrethroids performed substantially better than a model where these resistances did not interact. This indicated that associations among the efficacy of insecticides can be used to explain, in part, spatio-temporal variation in the susceptibility test observations.

The predictions of mean mortality across time and space show strong associations among the three insecticides in populations from both west and east Africa (Figure 4.4A-C). The Pearson correlation coefficient (r) between predicted prevalence of resistance to deltamethrin and that to permethrin had a posterior mode of rm = 0.91 (credible interval (CI) = 0.90, 0.93) and rm = 0.94 (CI = 0.94, 0.95) for west and east Africa, respectively. Mortality is slightly higher for Type II pyrethroids at intermediate levels of resistance in west Africa, as observed by many of the blue points being below the 1:1 line in Figure 4.4A and above the line in Figure 4.4C. No such pattern is seen in east Africa where most data sits on the 1:1 line. This difference between the regions could be used to support the hypothesis that

resistance to the two classes of pyrethroid could be diverging, though the evidence is relatively weak due to the lower number of data-points in the eastern region (especially from populations with high resistance) and because predictions were being made at a very large geographical scale. It should also be noted that perhaps differences in the diagnostic dose calibration for the different insecticides might only become apparent at the lower mortalities. This difference is less pronounced in recent analyses with a larger dataset(seen by similar ranges across both insecticides, Figure 4.4D-I, Hancock *et al.*, 2020), though this study was not intended to compare differences within pyrethroids so further work is required to test this hypothesis.

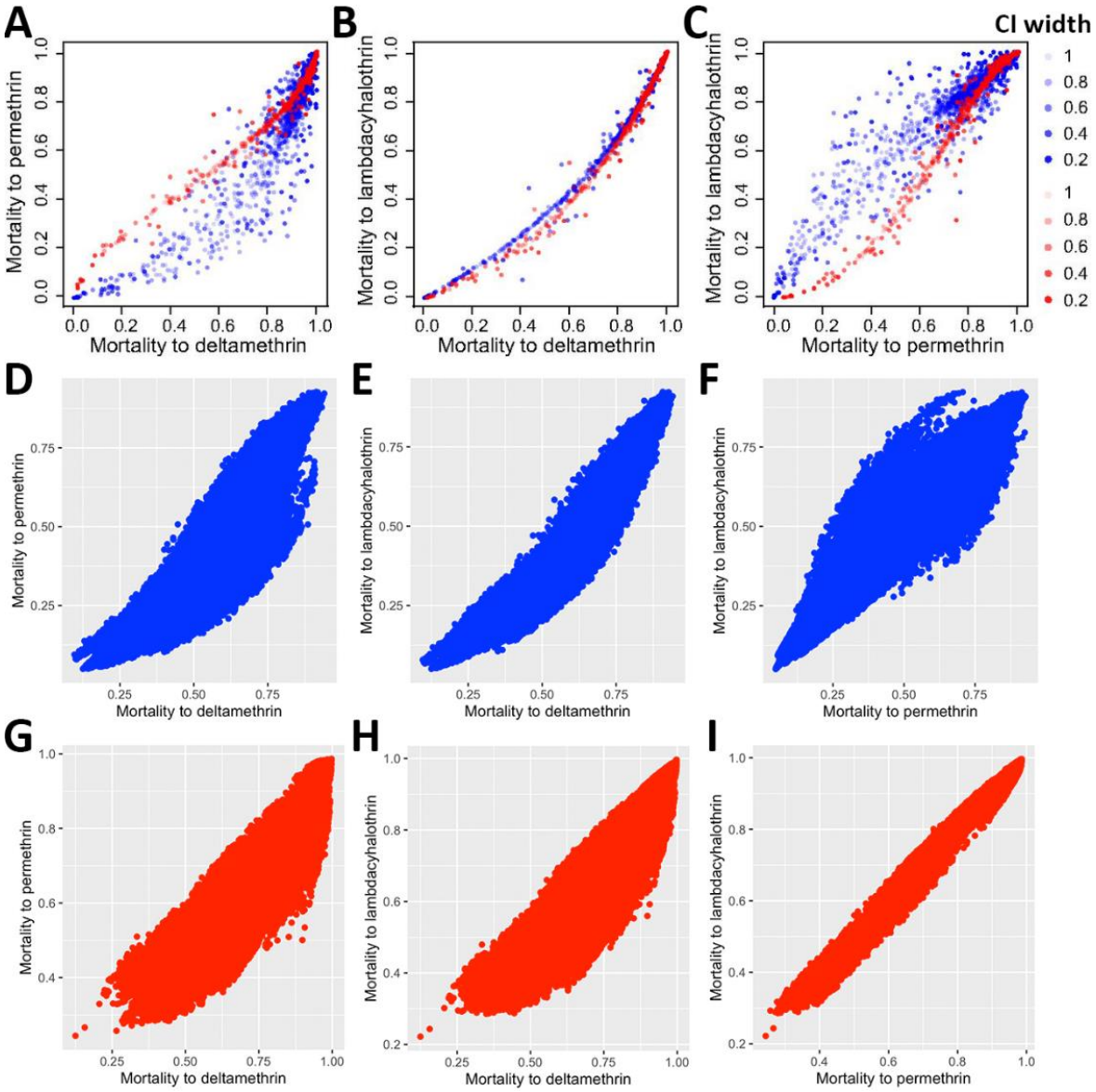


Figure 4.4. Associations among predicted resistance to three pyrethroids. Colour intensity indicates the width of the credible interval (CI) of the predicted mean for the data points shown in panels A-C. Panels A-C: Results from the 2018 study (Hancock *et al.*, 2018) with the west Africa results shown in blue and the east in red. Panels D-F: Results from the west Africa model used in the 2020 study (Hancock *et al.*, 2020). Panels G-H: results from

the east Africa model used in the 2020 study. Plots are shown for 2017 as this year had the best spread of mortality values.

More recent work added α-cypermethrin susceptibility data to the pyrethroid data previously included and used potential explanatory variables (covariates) such as ITN coverage, agricultural data and climate data in an ensemble of machine learning and geostatistical models (Hancock *et al.*, 2020). This study did not explicitly aim to test associations among resistance to different pyrethroids but did again find that a joint model performed best, indicating associations among the spatio-temporal trends in resistance to these four pyrethroids. These models produced finer resolution data giving higher numbers of mean predictions, so we have selected a single year to plot in Figure 4.4D-I. The pairwise associations between predicted resistance to α-cypermethrin and the other pyrethroids are shown in Figure 4.5.

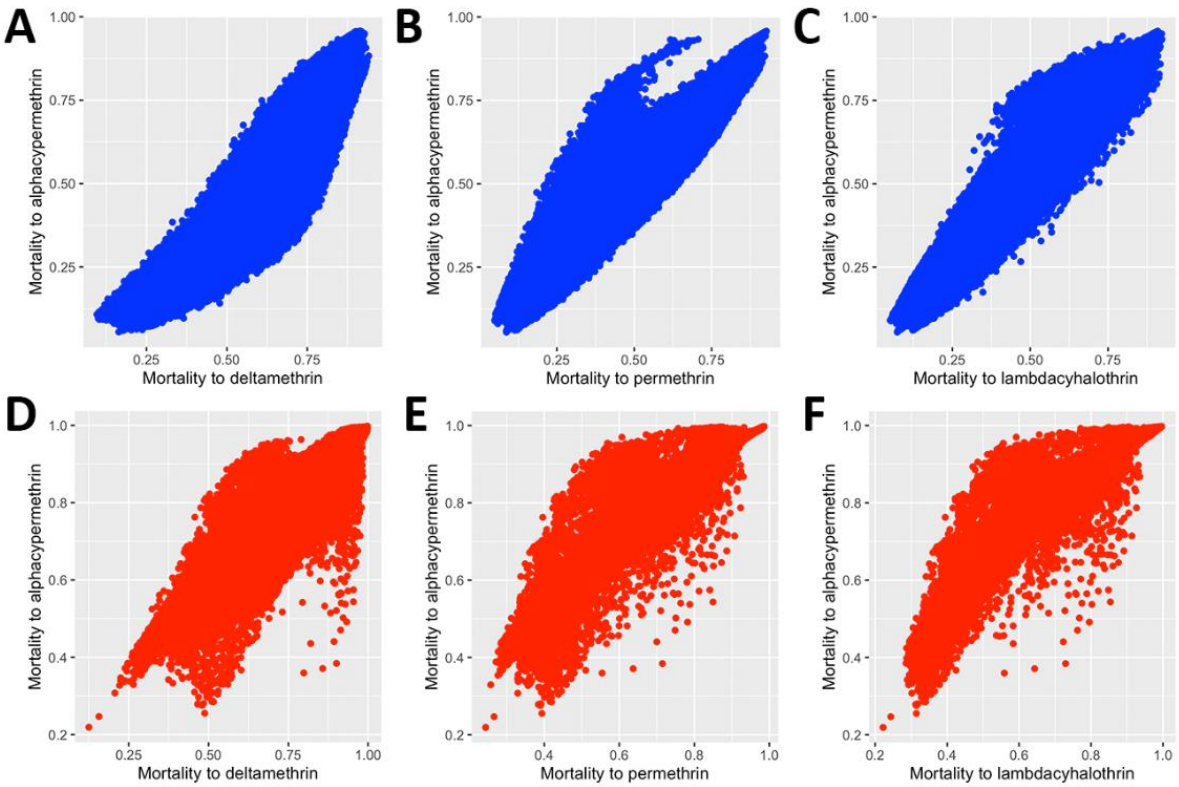


Figure 4.5. Associations among predicted resistance to four pyrethroids. Panels A-C: Results from the west Africa model used in the 2020 study. Panels D-F: Results from the east Africa model used in the 2020 study. Plots are shown for 2017 as this year had the best spread of mortality values.

4.2.4. Resistance intensity bioassays

Illustration of different resistance intensity bioassays conducted in LITE with highly inbred mosquito colonies are provided in Figure S4.1. Here, we use data collected with wild mosquito populations

which have greater variability in the shape of their dose response curves and are potentially conducted in more variable field laboratories. This dataset consists of 917 resistance intensity bioassays (2,966 individual replicate mortality values) from 23 African countries (Table S4.1), over half of the bioassays being conducted in either Nigeria and Mali. On average, each assay had mortality estimates from 2.8 different doses (Figure S4.2), with, on average, 82 mosquitoes exposed to the insecticide for each dose (when it was recorded). There were only 241 bioassays where permethrin and deltamethrin were directly compared in the same experiment. Given the variability observed in the discriminating dose bioassay this appears relatively low so data-points were clustered annually into regions no more than 50 km in diameter to increase the quantity of data which could be utilised. The location of the different individual data locations and how they are grouped into clusters are shown in Figure S4.3. This generated a total of 278 clusters (from 165 locations) which had resistance intensity data for permethrin (a total of 440) and deltamethrin (431, Figure 4.6A, Table S4.1). The two most common insecticides of each class were permethrin and deltamethrin, so analyses were restricted to these two insecticides. Generalised Mixed Effects Linear Models were used to estimate the impact of insecticide dose on mosquito mortality. Different shaped functions describing the dose response curve were tested on laboratory and field data. Results are presented as LD_{50} estimates and predictions of mortality at the discriminating dose concentration generated using the dose response model. This later readout can be considered more accurate than analysing raw discriminating dose data alone due to the repeated number of replicates in this assay.

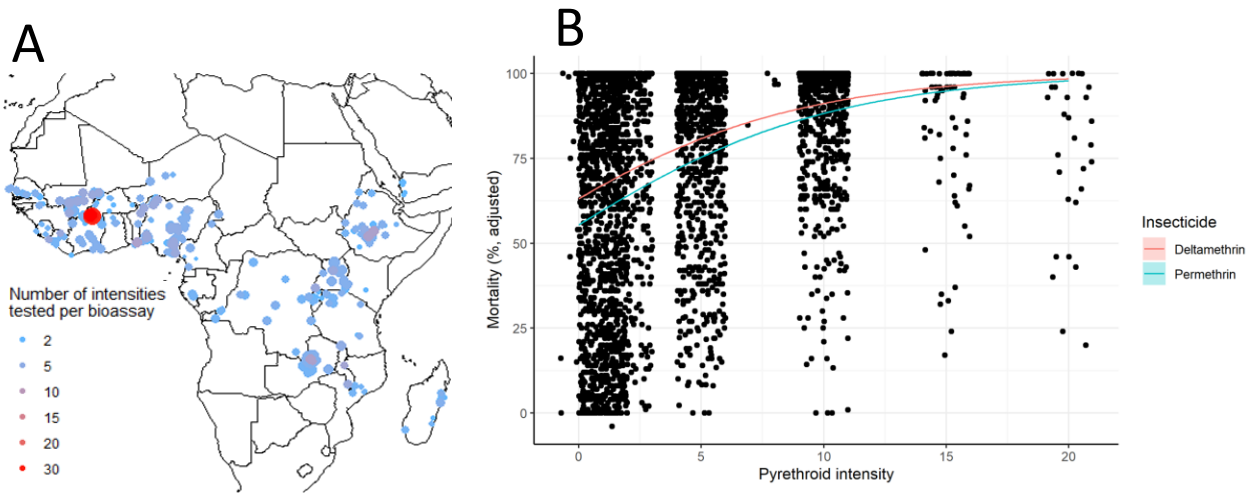


Figure 4.6. Summary of the resistance intensity bioassay data used in the analysis. (A) The geographical spread of data across Africa. Each point indicating a different intensity assay. (B) plot of all raw data used in the analysis (summarised in Table S4.1). Variability (jitter) is added to the points on the x-axis to make it easier to differentiate results from individual bioassays. Coloured lines indicate a simple dose-response curve fit to all data for the two different insecticides.

Overall, mortality shows a clear dose response curve for both permethrin and deltamethrin (Figure 4.6B). Mortality induced by deltamethrin was significantly higher than permethrin ($p<0.0001$). This is consistent with results from the discriminating dose bioassay and the difference persists across the range of insecticide concentrations tested here (Figure 4.6B). This overall consistently-shaped dose response curve is compatible with the hypothesis that the two insecticides have different discriminating doses (as proposed in section 3). If the concentration of permethrin selected for the original discriminating doses induces a higher level of mortality than that selected for deltamethrin then this discrepancy will be propagated across all concentrations (as they are relative to the discriminating dose at 2X, 5X and 10X; section 4.1). As a result, deltamethrin mortality will be higher across the range of concentrations as has been observed here.

When analysing data at the country level different trends are observed in different countries. Most countries where data are available report higher levels of deltamethrin mortality, with lower average LD_{50}S estimates (Figure 4.7 provides illustrative examples of 3 countries whilst further plots are provided in Figure S4.4). Lower pyrethroid divergence is observed in countries with lower levels of resistance (for example, Madagascar Figure 4.7A). The major exception is Zambia where dose response curves indicate that deltamethrin induces lower mortality and has a lower LD_{50} (Figure 4.8), which may reflect the species composition and the presence of specific target site mutations.

If there were some difference in the suite of resistance mechanisms for Type I and Type II pyrethroids and these mechanisms were established in populations, then it might be expected that resistance would diverge over time if selection pressures were consistent. Selection is thought to be driven, at least in part, by ITN use, so this selection pressure is likely to be relatively consistent as ITNs are typically replaced every three years. Differences in time are hard to observe from these resistance intensity data as results are only available for 1-4 years. Nevertheless, results are surprisingly consistent over the different years with those countries showing differences between pyrethroids generally persisting (Figure 4.7 and 4.8). Discriminating dose bioassay data has shown a clear increase in resistance over time (Hancock *et al.*, 2020). This trend is not evident in the resistance intensity data analysed here, with some countries, such as Malawi, showing a significant increase in resistance and others showing a significant decrease (Table 4.1). In Ethiopia and Mali there appears to be a significant increase in deltamethrin resistance and a decrease in permethrin resistance and it would be interesting to investigate what type of ITNs were used in these locations over the time-period. On average differences in mortality between insecticides does not increase over time, providing support for the cross-resistance hypothesis.

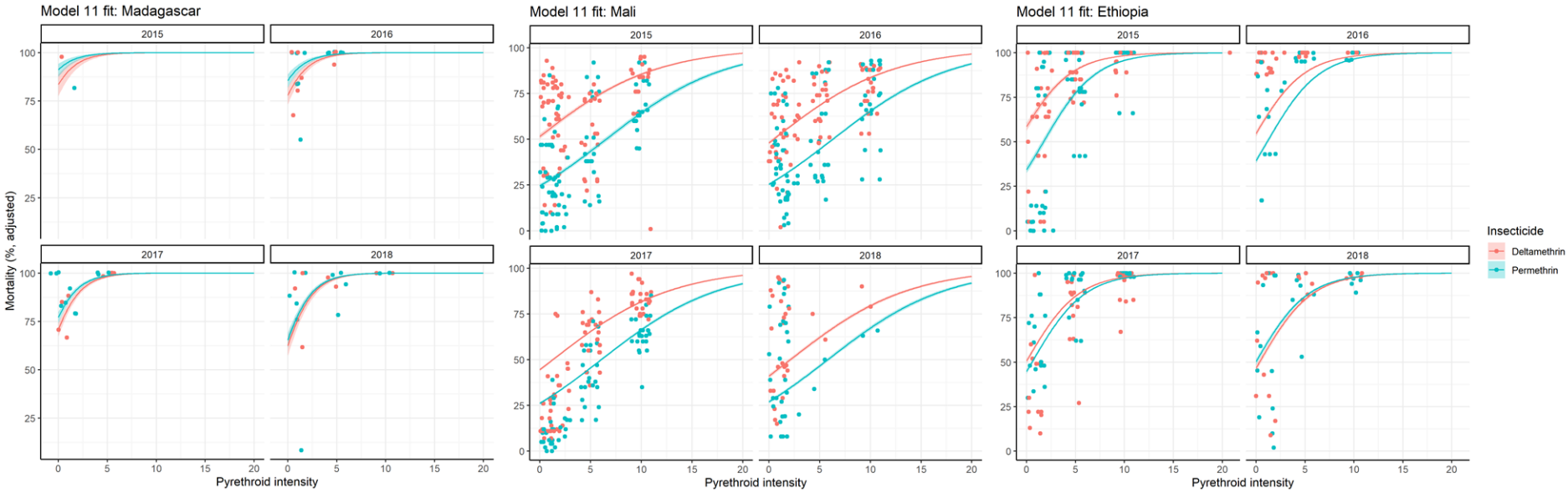


Figure 4.7. Dose response curves for different countries with data over 4 years from 2015-2018. Red points indicate mosquito mortality estimates observed in different clusters induced by either permethrin (green) or deltamethrin (red) for different insecticide concentrations (1 being the discriminating dose concentration). Coloured lines indicate the best fit dose response curve which was allowed to vary between insecticides and over time. Individual assays are grouped together in clusters (Table 4.1), pooling data from the same calendar year which were conducted within a 50km radius (Figure S4.3). Results are shown for (A) Madagascar, (B) Mali and (C) Ethiopia.

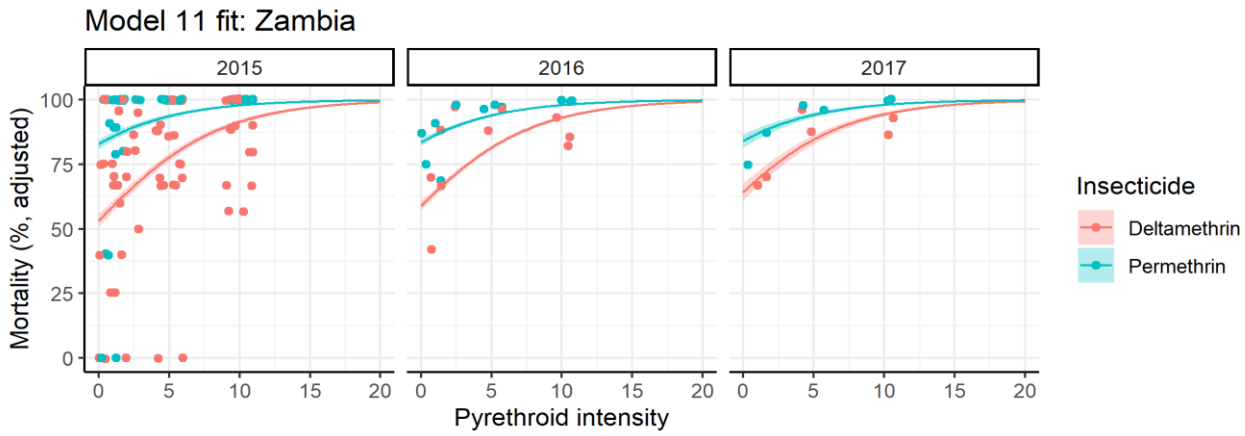


Figure 4.8. Dose response curves for Zambia which shows a qualitatively different trend to models from other countries with higher permethrin mortality. Red points indicate mosquito mortality estimates observed in different clusters induced by either permethrin (green) or deltamethrin (red) for different insecticide concentrations (1 being the discriminating dose concentration). Coloured lines indicate the best fit dose response curve which was allowed to vary between insecticides and over time. Individual assays are grouped together in clusters (Table 4.1), pooling data from the same calendar year which were conducted within a 50km radius (Figure S4.3).

Importantly, a significant difference between insecticides is not necessarily substantial enough to have a meaningful public health impact. The absolute difference in mortality at the discriminatory dose predicted by the dose response model is relatively low, varying from between 2-27% between countries with multiple years of data (Table 4.1). Temporal trends, when they do appear, are also relatively minor, changing by on average only a small percentage over the timeframe (these changes are significant as there is a lot of data but the change is not substantial). Care should be taken when interpreting absolute discriminating dose differences alone as differences will naturally be very low in highly resistant mosquito populations (Section 3). Here differences are seen when the average level of resistance is at intermediate levels suggesting this analysis remains valid. The shape of the dose response curves is broadly consistent across the different insecticides. There is some evidence of slight differences in the shape of the dose response curves for the two insecticides in some countries, though patterns are not consistent between regions and there are insufficient data to justify inclusion within the final dose response curves presented in Figure 4.6-8.

The average difference in mortality between permethrin and deltamethrin may be relatively low though national trends may hide substantial sub-national variation. Cluster analysis shows there is substantial regional variation in the LD_{50} estimates (Figure 4.7). Whilst results appear relatively consistent across Mali, there is considerable sub-national heterogeneity within DRC, Ethiopia, Nigeria and Zambia (which showed an opposite overall trend to the other countries). Much of this variation is

at fine geographical scales, and therefore substantially below the level at which ITN allocation decisions are made. With the current granularity of data it is unclear whether this fine scale heterogeneity is a result of true differences in the mosquito populations or a consequences of variability in the assays. Analysis of laboratory data shows a relatively low measurement error (RMSE = 5-9%, Figure S4.5). The clustering analysis shows substantially high levels of variation within 50km regions (RMSE = 9-28%, Figure S5.5 and S5.6) indicating the problems for differentiating between variability and true differences. Nevertheless, the relatively consistent error reported in the field between permethrin and deltamethrin resistance intensity assays and across the range of resistance levels suggests local differences are driven by assay variability, and mosquito population composition, rather than true underlying differences between pyrethroid classes.

Table 4.1 Summary of the temporal results for countries with data recorded over multiple years. The second and third column indicate whether there was evidence of a significant increase or decrease in resistance over time (white boxes indicate no significant change). Fourth column indicates the average difference in the discriminatory dose concentration (predicted by the dose response model) between the two insecticide (negative values indicating greater deltamethrin mortality) whilst subsequent columns show the breakdown for the different years where results were available.

Country	**Resistance change over time**		**Difference between permethrin vs deltamethrin mortality at 1x intensity (%)**				
	Permethrin	**Deltamethrin**	**Mean**	**2015**	**2016**	**2017**	**2018**
DRC	↓		-27			-35	-20
Ethiopia	↓	↑	-11	-25	-36	22	-10
Uganda	↓	↓	-18	-28	-9		
Zambia		↓	12	10	17	12	
Madagascar	↑	↑	-2	-16	0	6	-9
Nigeria	↓	↓	-11		-5	-21	-10
Mali	↓	↑	-22	-35	-22	-13	-13
Malawi	↑	↑	-8			-11	-4

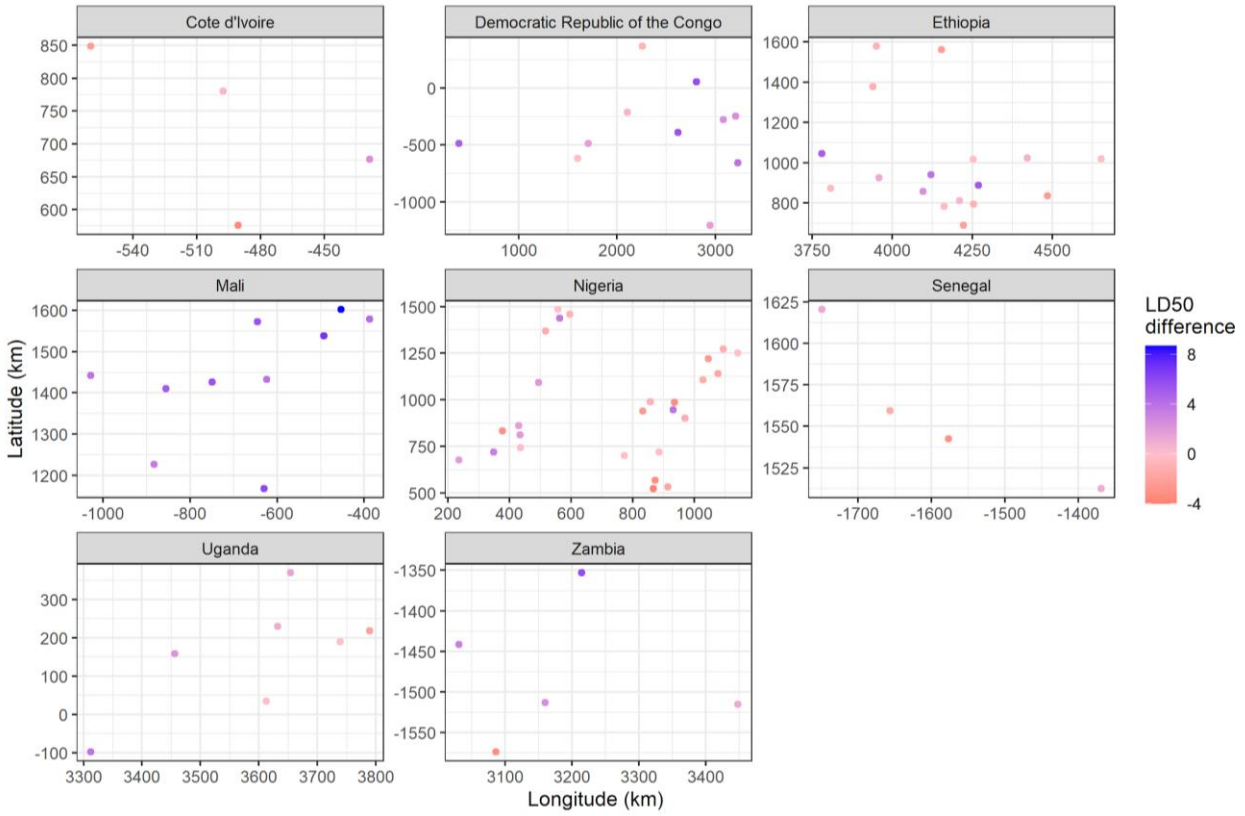


Figure 4.7. Cluster-level data for the difference between permethrin and deltamethrin LD_{50}. Point show the geographical location of the different clusters in each country. Each cluster has a maximum distance of 50km between all sampling locations. Colour denotes the average difference in permethrin and deltamethrin induced mortality within each cluster, a positive LD_{50} difference (blue colours) indicates a higher LD_{50} for permethrin (greater permethrin resistance, lower permethrin mortality).

4.2.5. Experimental hut trials

The results of the experimental hut trial meta-analysis are consistent with the discriminating dose and resistance intensity bioassay data. Fourteen studies were identified which compared bednets treated with Type I and II pyrethroids, which contained a total of 28 replicates that could be directly compared (Table S4.2). On average Type II insecticidal bednets had slightly higher levels of mortality than Type I (Figure 4.8). This was observed across a range of insecticide resistance levels, though care should be taken as many of the studies reporting high mortality were with conventionally treated nets and not ITNs. Similarly, there is no evidence of differences in other behaviours which can be measured in hut trials, such as blood-feeding or deterrence. This suggests that both types of ITNs may have similar epidemiological benefits. It is also interesting to note that, consistent with the resistance intensity bioassay, the difference between Type I and Type II nets seems consistent across the range of

insecticide resistance levels. As previously highlighted, it could be argued that greater variation would be expected at higher levels of resistance if Type I and Type II pyrethroids had different resistance mechanisms. The presence of different resistance mechanisms would provide a mechanism by which different selection pressures between sites could act to drive divergence of resistance as there would have been greater opportunity for susceptibility to vary between sites if there were different selection pressures.

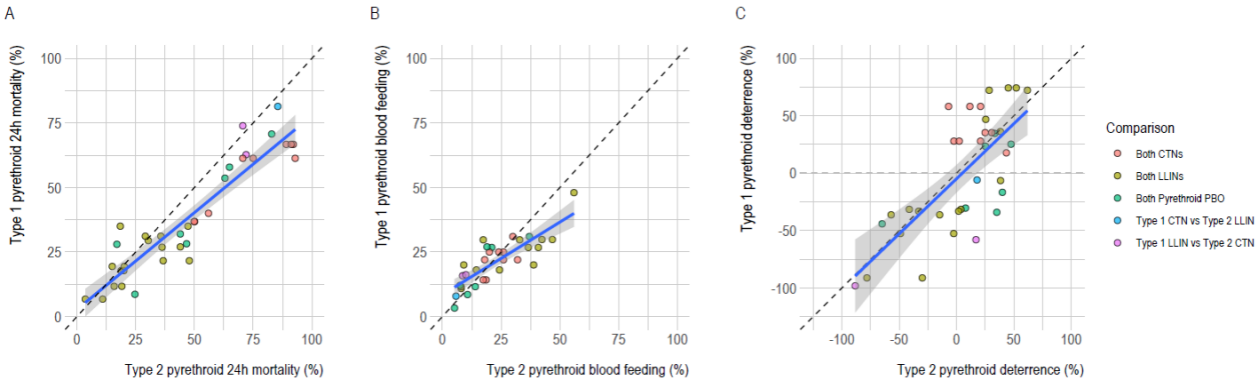


Figure 4.8. Summary of experimental hut trials comparing Type I and II pyrethroids identified in the systematic review. (A) the level of 24-hour mortality (B) blood-feeding and (C) deterrence (the propensity for mosquitoes to be caught in the hut with the pyrethoid bednet compared to a hut with an untreated control net) as recorded in the hut trial. Point colour indicates the type of bednet evaluated, be it conventionally treated net (CTN), a pyrethroid only long-lasting insecticidal net (ITN) or a pyrethroid and PBO ITN.

4.2.6. Conclusions

- The resistance intensity bioassay represents a substantial improvement over the discriminating dose bioassay in areas with moderate and high insecticide resistance. However, it is prone to substantial sampling and measurement error so results from individual data sets should not be overly interpreted and should be analysed together in a robust statistical framework to understand long-term trends.
- Evidence from discriminating dose bioassays, resistance intensity bioassays and experimental hut trials all indicate, on average, slightly higher mortality to Type II than Type I pyrethroids in wild mosquito populations. Since the discriminating dose for permethrin and deltamethrin may induce slightly different levels of mortality (*Evidence from laboratory strains (a)* point 3), so it is not clear whether these differences in mortality reflect true differences in resistance. Nevertheless, true difference of this level is unlikely to have a substantial public health impact, especially when other intrinsic differences between ITNs, such as the surface bioavailability of insecticide, are considered.

- There is no evidence of divergence of mortality over time induced by Type I and II pyrethroids in field mosquito populations, i.e. the average difference between pyrethroids has remained consistent over time rather than increasing as might be expected if substantial differences in phenotypic resistance were selected for a prolonged period.
- The variability in discriminating dose and intensity assay mortality is high. This variability is predominantly at a local geographical scale indicating that if there were a difference between Type I and II pyrethroids it will be beneath the size of the region of deployment for ITNs or IRS.

4.3. Investigating whether differences between pyrethroids identified by molecular studies (Section 2) can be detected in wild mosquito populations

C. L. Moyes, P. A. Hancock and H. M. Ismail

The review of molecular studies in Section 2 provides evidence that some pyrethroids are closely related in terms of their vulnerability to metabolic attack whereas others are more divergent. In this section (4.3), we analyse field data to investigate whether the similarities and differences identified by molecular studies are also detected by field studies of resistance within African malaria vector populations, bearing in mind the calibration issues and sources of variability in the data described in previous sections.

One of the compounds identified in Section 2 as being of interest, bifenthrin, has not been included in studies of resistance in African populations. We therefore also review a small number of studies that have investigated resistance to this compound compared to other pyrethroids in *Anopheles sinensis* and *Aedes aegypti* populations.

4.3.1. Methods

4.3.1.1. Relative divergence in resistance to pyrethroids within wild populations of malaria vectors

Data are available on the prevalence of the insecticide resistance phenotype in African malaria vector populations, from routine monitoring surveys and from research studies that use one of two standard methodologies: the WHO insecticide susceptibility test or the CDC bottle bioassay. Data from bioassays that exposed mosquito samples to standard diagnostic doses were extracted from a published database (Moyes *et al.*, 2019). All instances where a single *An. gambiae* s.l. sample was subdivided and the different subsets exposed to different pyrethroids were identified, and paired datasets were created for all combinations of α-cypermethrin, cyfluthrin, deltamethrin, etofenprox, λ-cyhalothrin and permethrin. Paired samples were selected (rather than data from studies that had, for example, tested 3 or 4 pyrethroids against the same mosquito population) because there were insufficient data from studies testing >2 pyrethroids. Only data that detected some level of pyrethroid resistance in the full mosquito sample were included. That is, results from samples that had 100% mortality to all compounds tested were excluded. The final dataset contained mortality values for 3,432 pyrethroid pairs from 961 sites in 33 African countries from 1995 to 2018 (Table 4.2).

Correlation analyses were performed to identify which pairs of pyrethroids are most similar or divergent in terms of the resistance found to them within *An. gambiae* s.l. populations. The lowest number of available data points was 20 (Table 4.2), so the initial correlation analysis used 20 randomly selected data points for each pyrethroid pair and calculated the Pearson's correlation coefficients using SPSS Statistics v25. Most of the results (9/15) were not significant (at the 0.05 level with a Bonferroni adjustment) indicating that a sample size of 20 was not sufficient to detect the correlations that we wish to compare.

In the final analysis, we used a sample size of 42, which yielded significant correlations for each pair of pyrethroids, and we excluded 2 pyrethroid combinations that did not have 42 data points (cyfluthrin vs etofenprox and cyfluthrin vs α-cypermethrin).

Table 4.2. Number of susceptibility test pairs. Pairs of results where mortality to at least one pyrethroid was <100%.

	Deltamethrin	**Permethrin**	**λ-cyhalothrin**	**α-cypermethrin**	**Etofenprox**	**Cyfluthrin**
Deltamethrin	NA	1278	597	243	81	65
Permethrin		NA	484	197	68	62
λ-cyhalothrin			NA	154	74	54
α-cypermethrin				NA	42	27
Etofenprox					NA	20
Cyfluthrin						NA

Forty-two data points were selected randomly from the full dataset for each pyrethroid combination, and Pearson's correlation coefficient was calculated for each pair. This was repeated 20 times and a mean correlation coefficient values calculated for each pyrethroid pair. These values were then ranked to identify the pairs that were most similar or divergent in terms of their resistance prevalence in *An. gambiae* s.l. mosquitoes. Finally, the correlation coefficients were used to generate a dendrogram using the unweighted pair-group method with arithmetic mean (UPGMA), where the highest correlation coefficient indicated the most similar pair. This dendrogram was then compared to those generated from molecular studies as detailed in Section 2.

4.3.1.2. Associations in the spatio-temporal trends in resistance to different pyrethroids within malaria vector populations

Much larger volumes of data are available for resistance to each pyrethroid individually. The times and locations of the *An. gambiae* s.l. collections used differ for each of the pyrethroids tested, meaning direct comparisons (like those described above) are not possible using the full dataset. Instead, our 2018 study used a geostatistical model that distinguished the spatial and temporal trends in the susceptibility test data from noise in these data and then compared the trends detected for each pyrethroid (Hancock *et al.*, 2018). This approach requires large volumes of data and was thus restricted to testing associations among resistance to deltamethrin, λ-cyhalothrin and permethrin, and to DDT. The model did not use covariate data. Two sets of models were trained on the susceptibility test data independently: one using west Africa data and one using east Africa data, reflecting the two regions with sufficient data. For each region, linear models of coregionalization were used to test the predictive power of jointly modelling resistance to multiple pyrethroids compared to modelling resistance to each compound independently. That is, were we better able to predict deltamethrin resistance by jointly modelling all three pyrethroids or by training the model using deltamethrin data alone, and so on. All models were validated using a subset of the field data that was withheld from the model. For instances where the joint model performed best, we then tested the correlations among the predicted resistance values for each of the pyrethroids included in that model. These correlations tested the relationships between the spatio-temporal trends in the prevalence of resistance to each pyrethroid, excluding data noise, whereas the analyses described in the above section using paired results from the same samples and did not exclude data noise.

4.3.1.3. Quantitative structure-activity relationship of pyrethroids against wild populations of other vectors

Published quantitative data of pyrethroid susceptibility profiles from wild populations of *Anopheles sinensis* and *Aedes aegypti* were collated and cleaned for quantitative structure-activity relationship (SAR) analysis. QSAR analysis carried out by clustering the resistance ratio based on median knockdown concentrations (KC_{50}) and median lethality concentrations (LC_{50}) data for pyrethroids recommended by WHOPES (Section 2 Figure 2.1) to understand the impact of structural diversity on pyrethroid resistance/susceptibility. Also, consideration has been given to piperonyl butoxide (PBO) inhibition data to estimate the contribution of mixed-function oxides mainly cytochrome P450s in pyrethroid resistance/susceptibility.

4.3.2. Results

4.3.2.1. Relative divergence in resistance to pyrethroids within wild populations of malaria vectors

Significant correlations were found for all pairs of pyrethroids tested using data from *An. gambiae* s.l. samples collected across Africa (at the 0.05 level with a Bonferroni correction, n=42 samples collected across Africa for each pair). The resulting Pearson's correlation coefficient values were compared to identify which pairs of pyrethroids are most similar or divergent in terms of resistance within *An. gambiae* s.l. communities. The ranked correlation coefficients (Tables 4.3 – 4.4) show that deltamethrin and λ-cyhalothrin were the most closely correlated pair whereas associations with etofenprox were more divergent. Caution should be used when interpreting the exact ranking of the pairs of pyrethroids for which there was a limited pool of data from which to draw 42 randomly selected data points.

The resulting dendrogram shows that the hierarchical relationships among these pyrethroids are similar to those found using data on the inhibition of these compounds by cytochrome P450 enzymes, indicating that similarities and divergence among pyrethroids identified by molecular studies can be detected in field studies (Figure 4.9 and Section 2 Figure 2.1B). This isn't a perfect match and exact alignment between the molecular and field studies was not expected because there are additional variables that influence resistance in the field including other mechanisms of resistance.

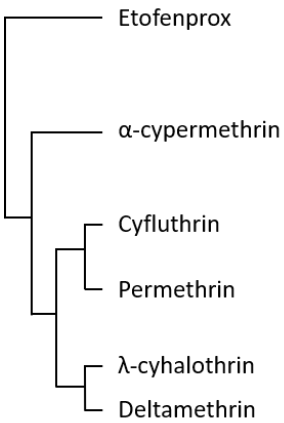


Figure 4.9. The hierarchical relationships among six pyrethroids in terms of resistance to each compound in African malaria vector populations.

The analyses conducted using data from *An. gambiae* s.l. samples were repeated using data from *An. funestus* subgroup samples as well as results for *An. arabiensis*, *An. coluzzii*, *An. funestus* and *An. gambiae* populations. There were much lower data volumes for the individual species and a limited selection of pyrethroid pairs could be tested so no dendrograms were constructed from these data.

Across *An. funestus* subgroup communities, there were significant correlations between resistance to deltamethrin and λ -cyhalothrin, permethrin and λ-cyhalothrin, and deltamethrin and permethrin (Table S4.7) and the same was true for the species tested (Table S4.8). There was insufficient data to test the other pyrethroid combinations. 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 wider context of cross-resistance. Significant correlations with the prevalence of resistance to DDT were found for species with the *An. gambiae* complex but not for *An. funestus* (Table S4.9). This matches our knowledge of the mechanisms of resistance found in these species. Mutations to the VGSC gene (*kdr* mutations) are known to confer resistance to both pyrethroids and DDT and are common in species of the *An. gambiae* complex but have not been found in *An. funestus* or other members of the *An. funestus* subgroup (Kawada *et al.*, 2011). No significant correlations were found between the prevalence of resistance to deltamethrin and that to bendiocarb and propoxur (carbamates), malathion, fenitrothion and pirimiphos-methyl (organophosphates). This underlines the point that it is cross-resistance within the pyrethroids, as well as between the pyrethroids and DDT, that is most important within wild malaria vector populations.

These results provide information about associations between resistance to different pyrethroids within each species, however, these data do not provide information about differences between species. An ongoing project is investigating pyrethroid resistance in sympatric populations and the preliminary data show that while there are differences among species in the prevalence of resistance (*An. gambiae* > *An. funestus* > *An. arabiensis*) where they co-occur, there also appears to be a general trend that in locations where pyrethroid resistance is higher in one species it is also higher in the others (Figure S4.7). The data that are currently available are insufficient to allow a simultaneous analysis of pyrethroid effects, species effects, and the interactions between them.

4.3.2.2. *Associations in the spatio-temporal trends in resistance to different pyrethroids within malaria vector populations*

The 2018 study found strong associations among the spatio-temporal patterns of resistance to all three pyrethroids tested and the results are given in full in Hancock *et al.* (2018). The posterior mode rm for the Pearson's correlation coefficient r between the predicted mean prevalence of resistance for each pyrethroid pair for west and east Africa showed that deltamethrin and λ-cyhalothrin are the most closely correlated pyrethroid pair and the correlation values for the other two pairs, deltamethrin vs λ-cyhalothrin and permethrin vs λ-cyhalothrin, were very similar to each other (Tables S4.5 and S4.6). This provides further validation of the results presented in Figure 4.9 and Tables S4.3 and S4.4, for these three pyrethroids.

4.3.2.3. Relative divergence in resistance to a wider range of pyrethroids within wild populations of *Aedes aegypti* and *Anopheles sinensis*

Cross-resistance data relevant to the pyrethroids investigated by the molecular studies (Section 2) were also extracted from Flores *et al.*, (2013) to evaluate the relative divergence in resistance to pyrethroids within wild populations of *Aedes aegypti*. Seven F1 wild strains of *Aedes aegypti* (L.) from northern eastern Mexico were evaluated by bottle bioassay for resistance to permethrin, bifenthrin, deltamethrin, α-cypermethrin and λ-cyhalothrin. The resistance ratio relative to the New Orleans strain of 50% knockdown values ($RRKC_{50}$) and 50% lethality values ($RRLC_{50}$) are illustrated in Table S7 and Table S8, respectively. Generally, the $RRKC_{50}$ indicates high levels of knockdown resistance. The $RRKC_{50}$ from α-cypermethrin varied from 10 to 100 among wild populations indicating high levels of knockdown resistance (Flores *et al.*, 2013). Significant but much lower levels of resistance were detected for λ-cyhalothrin and permethrin. For bifenthrin, only one wild population exhibited resistance with $RRKC_{50}$ values about 21-fold. None of the population showed $RRKC_{50}$ >10 with deltamethrin, and only moderate resistance was seen in three populations, while the rest were susceptible (Flores *et al.*, 2013). Regression analysis used to analyse the relationships between the pyrethroids for $RRLC_{50}$ and $RRKC_{50}$. All the pyrethroids were highly correlated (in terms of both $RRKC_{50}$ and $RRLC_{50}$) except bifenthrin, indicating strong cross-resistance (Figure 4.10A and B). The resistance profiles were highly correlated for deltamethrin and λ-cyhalothrin (p= 0.924), followed by permethrin with α-cypermethrin (p= 0.795) Figure S4.8. The resistance values for bifenthrin were not correlated with any of the other four compounds (Figure S4.10). A similar finding was observed with the wild population of South Korean malaria vector *Anopheles sinensis* which was highly susceptible to

bifenthrin followed by cyfluthrin (Chang *et al.*, 2013). Overall, biological data available from both *Aedes aegypti* and *Anopheles sinensis* suggest that bifenthrin can be used as alternative pyrethroid without strong cross-resistance to permethrin. This is likely due to the low similarity of bifenthrin with other pyrethroids tested in both studies.

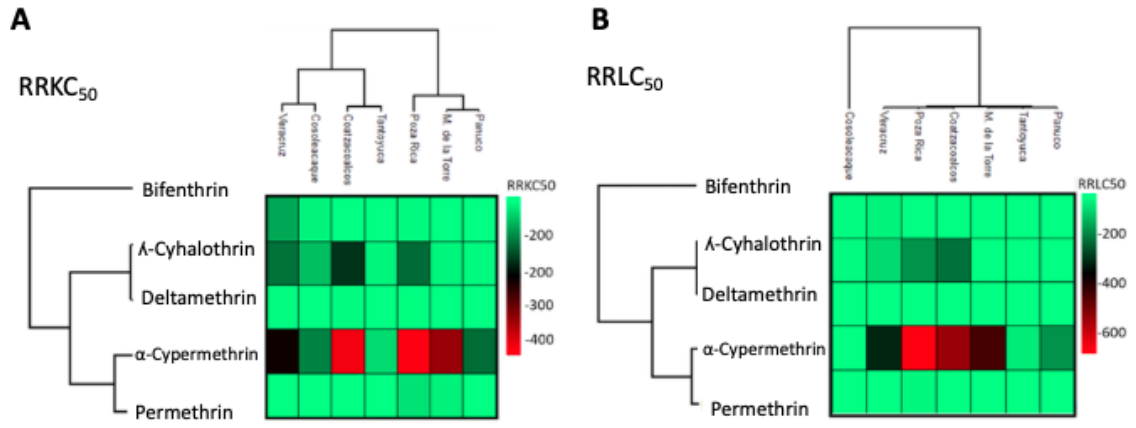


Figure 4.10 Dendrograms arising from cluster analysis of the *Pearson* correlation coefficient for (A) $RRKC_{50}$ and (B) $RRLC_{50}$ for resistance to each of the five pyrethroids in *Ae. aegypti* strains from Mexico.

4.3.2.4. Can increased vulnerability to enzyme attack found in molecular studies be detected in wild mosquito populations?

The molecular evidence reviewed in Section 2 reveals that some pyrethroids are more vulnerable to cytochrome P450 attack, particularly etofenprox, and it is therefore interesting to consider whether resistance to this compound is higher in wild mosquito populations. Caution is needed in interpreting differences found using susceptibility test data because of the calibration issues described in Section 3, however, an analysis of the paired data from *An. gambiae* s.l. samples collected across Africa provides no evidence that the prevalence of resistance is higher for etofenprox compared to the other pyrethroids (Figure S4.9).

4.3.2.5. Pyrethroids showing increased resistance (decreased mortality) in African malaria vector species

The prevalence of resistance to permethrin in *An. gambiae* s.l. was typically found to be higher (lower mortality) than that to deltamethrin in the earlier geostatistical study (Hancock *et al.*, 2018), while the analyses in Section 4.2 of this report found that while differences between these two pyrethroids persisted across dose intensities, the relationship was reversed in some geographical regions when

data from all species and complexes were analysed. Caution is needed in interpreting differences found using intensity and susceptibility test data because they may be due to real differences in the prevalence of resistance, or differences in the calibration of the diagnostic doses (section 4.2), or both. It is, however, interesting to consider whether the reversal of this relationship shown in section 4.2 is associated with species differences.

Paired-sample *t-tests* of the data from the *An. gambiae* complex, the *An. funestus* subgroup and individual species used in the above correlation analyses show that the prevalence of resistance to permethrin was significantly higher (i.e. mortality was lower) compared to deltamethrin for the *An. gambiae* complex and for species within this complex, but that the prevalence of resistance to deltamethrin was significantly higher in the *An. funestus* subgroup and in *An. funestus* (Figures S4.10 and S4.11). These results match those from the geostatistical study and may explain the reversals seen in the analysis on intensity data.

We also found that the prevalence of resistance to λ-cyhalothrin was higher than that to deltamethrin for *An. gambiae* s.l., in agreement with the geostatistical study, and the same relationship was seen for the *An. funestus* subgroup, but this relationship was not detected in individual species for which the data volumes were much lower (Figures S4.10 and S4.11).

We found no significant differences in resistance to permethrin and λ-cyhalothrin in *An. gambiae*, again in agreement with the geostatistical study, but the prevalence of resistance to λ-cyhalothrin was significantly higher for the *An. funestus* subgroup. No significant differences were detected within individual species for which the data volumes were again much lower (Figures S4.10 and S4.11).

To put these results for difference within the pyrethroids into the wider context of the range of insecticide classes available for malaria control, the differences in the prevalence of resistance to deltamethrin were compared to the prevalence of resistance to six non-pyrethroid insecticides (Figure S4.12). 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 while *An. funestus* had significantly higher resistance to deltamethrin (Figure S4.12). In all species tested, the prevalence of resistance to deltamethrin was higher than that to bendiocarb and propoxur (carbamates), malathion, fenitrothion and pirimiphos-methyl (organophosphates) and these difference are much larger than those seen within the pyrethroids.

4.3.3. Conclusions

- There is good evidence that resistance to deltamethrin, permethrin, α-cypermethrin and λ-cyhalothrin are strongly correlated across *An. gambiae* s.l. communities and environments. These correlations were also seen for resistance to deltamethrin, permethrin and λ-cyhalothrin (α-cypermethrin was not tested) in the *An. funestus* subgroup and in *An. arabiensis*, *An. coluzzii*, *An. funestus* and *An. gambiae*.
- Strong associations between resistance to deltamethrin, permethrin, α-cypermethrin and λ-cyhalothrin mean that switching between these compounds is not recommended.
- If individual susceptibility test results at a site show differences between deltamethrin, permethrin, α-cypermethrin and λ-cyhalothrin, it is likely that these are due to data noise and more evidence is required before a decision to switch is made.
- There is evidence that resistance to etofenprox could diverge from resistance to the more commonly used pyrethroids. The potential high vulnerability to metabolic attack by P450s shown by molecular studies might preclude an operational switch to etofenprox, but higher resistance to etofenprox, compared to the more commonly used pyrethroids, wasn't detected in field-collected *An. gambiae* s.l.
- The divergence between bifenthrin and the other pyrethroids identified by molecular studies was detected in terms of phenotypic resistance by a small number of studies of field-collected *An. sinensis* and *Ae. aegypti* populations from one site in Korea and seven in Mexico, respectively. However, more direct evidence for whether resistance is lower in targeted vector populations, compared to the other pyrethroids, is needed before a switch could be considered.

4.4. Detection of cytochrome P450 mechanisms of pyrethroid resistance using synergist bioassays

A recent study reviewed the evidence for the presence of P450-mediated resistance mechanisms[1] in African malaria vectors (Moyes *et al.*, 2020). They found that 79% (276/351) of the PBO synergist bioassay data for *An. gambiae* s.l. confirmed the presence of these mechanisms, as did 94% (34/36) of the data for *An. funestus*. They found variation in the increase achieved when PBO is used, from 10 to 100%, although noise in the data is to be expected because the results are a combination of two

[1] Defined by WHO in note i of Figure 3.1 of the 2016 test procedures (WHO, 2016) as a ≥10% increase in susceptibility test mortality after the addition of PBO.

bioassays. Of the districts with data, 92% (114/123) met the criteria for confirmed cytochrome P450-mediated pyrethroid resistance and 8% of those tested had no results that met these criteria. It is worth noting that 41 districts had multiple test results that gave conflicting outcomes of both greater than (presence) and less than (absence) 10% increase in mortality with the addition of PBO. That is, most districts (41/50) with one or more test results that did not meet the criteria to confirm cytochrome P450-mediated pyrethroid resistance also had a test result that did meet the criteria. This may indicate variation within district, noise in the test data, or a rapid spread of these mechanisms of resistance. Either way, there is clearly little definitive contemporary evidence for absence of elevated cytochrome P450 in any of the regions tested. When we consider the data for individual pyrethroids (Figure 4.11), each of the four pyrethroids tested conforms to these general trends. That is, most of the results for α-cypermethrin, deltamethrin, λ-cyhalothrin and permethrin show that, when mortality is <90% without a synergist then a greater than 10% mortality increase is seen with the addition of PBO.

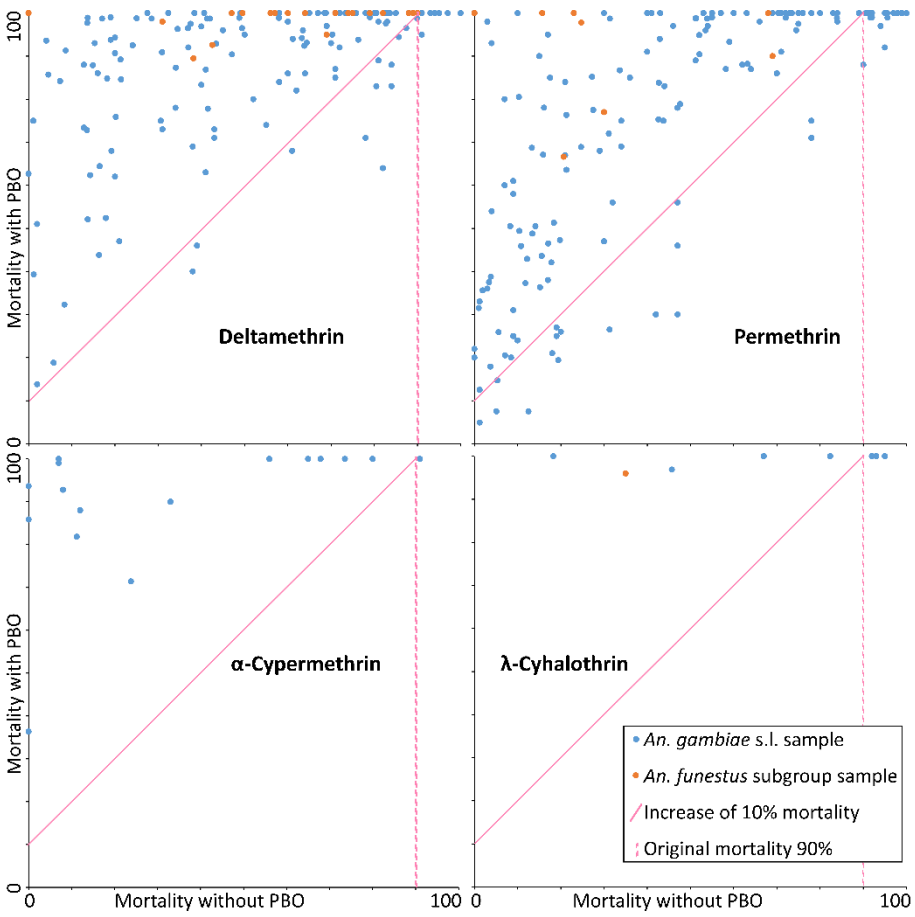


Figure 4.11. Plot of surveillance data from paired standard susceptibility tests using a pyrethroid (deltamethrin, permethrin, α-cypermethrin, λ-cyhalothrin) with and without the synergist PBO showing the full variation in the results and key thresholds defined in WHO guidelines.

5. Differences in behavioural responses to pyrethroids

P. J. McCall

5.1. The significance of behaviour in resistance monitoring

The mechanisms conferring resistance to insecticides include target site mutations that prevent binding of the insecticide and upregulation of the metabolic enzymes that detoxify it, as discussed in detail above. Less well known but also important resistance mechanisms include changes in the mosquito cuticle that reduce penetration of the insecticide, and behavioural resistance mechanisms such as shifts to exophily or exophagy in response to IRS and ITNs respectively, enabling mosquitoes to reduce contact with the insecticide. However, behavioural resistance may have many other less apparent forms and should be an important consideration in selecting a pyrethroid to deploy. If there are differences in behavioural responses to different pyrethroids, then behavioural resistance might diverge such that resistance to one pyrethroid might be overcome by deploying a different one, or deployment choice might need to consider whether certain pyrethroids are more or less likely to drive behaviour in resistant mosquitoes which will lower the ITN's efficacy. The discriminating dose and resistance intensity bioassays used to monitor resistance were never designed to include behavioural changes, and hence, behavioural resistance was not considered in this report until now. Yet a change in behaviour in a mosquito exposed to a pyrethroid may well confound the results of a bioassay, for example, an increase in sensitivity could reduce contact times at the treated surface resulting in lower mortality, whereas an increase in tolerance could increase the contact time and mask existing resistance.

5.2. The impacts of pyrethroids on mosquito behaviour

While the mechanisms of insecticide resistance in malaria vectors have been studied and characterised extensively at the molecular level, knowledge of behavioural changes associated with resistance is relatively poor. The primary purpose of pyrethroid insecticides delivered via IRS and ITNs is rapid killing of mosquitoes on contact, but many also have repellent properties. Mosquitoes detect insecticide residues on netting or solid walls by direct tarsal contact and respond by taking flight to break contact (see later section). However, as documented from many (but not all) experimental hut trials, pyrethroid residues on interior walls or on bednets can reduce *Anopheles gambiae* s.l. entry rates via the eaves. Although the processes by which pyrethroids deter mosquitoes without contact and at distances of 3 m or more are still not understood, it is an important aspect of bednet

performance. Deterrence can add benefit by reducing mosquito house entry rates, but it can also reduce contact with the insecticide, thus reducing the proportion of the vector population killed by ITNs, and hence the community or mass effect. Deterred mosquitoes may be diverted to unprotected humans. Differences in deterrence by different pyrethroids is a possible mechanism of divergent efficacy.

The behavioural responses of susceptible mosquitoes to insecticides had not been characterised in detail prior to the emergence of resistance in African malaria vectors and the arrival of next generation bednets. Since ITNs were first deployed, numerous experimental hut trials have recorded eave entry rates using different study designs and definitions of repellency to test different ITNs and hand-dipped nets, with vector populations at different locations on different dates, all during a period when pyrethroid resistance was emerging at variable rates across the continent. Not unexpectedly, given the diversity of wild populations studied and the uncharacterised variation in resistance status, the results are highly variable with little indication of any trend. Prior to the emergence of resistance, an early hut trial in the Gambia concluded permethrin was the most repellent, followed by λ-cyhalothrin, deltamethrin and lastly cypermethrin (Miller, Lindsey and Armstrong, 1991). Nearly a decade later in Côte d'Ivoire, Darriet *et al.* (2000) reported that deltamethrin-treated nets reduced *An. gambiae* hut entry rates by 72% in areas with susceptible populations but only 43% where vectors were resistant. In Benin, over 80% of *An. gambiae* s.s. with the *kdr* allele were easily repelled by high and low concentrations of permethrin, but no susceptible population was tested (Corbel *et al.*, 2004). Bifenthrin-treated nets reduced hut entry rates by over 80% in Cameroon where *An. gambiae* s.l. were resistant to pyrethroids, supporting the predictions from Section 2 of differential resistance to this pyrethroid, but again, no susceptible population was tested (Chouaibou *et al.*, 2006). Hougard *et al.* (2003) tested bednets with various combinations of bifenthrin and carbosulfan against both resistant and susceptible *An. gambiae* s.l. and reported no differences in entry rates between treatments or vector populations. Asidi *et al.* (2004) also tested bednets treated with α-cypermethrin, λ-cyhalothrin, permethrin, deltamethrin or carbosulfan against resistant *An. gambiae* s.l. in Ivory Coast. Here, all nets performed similarly with none exhibiting any deterrent effects until they had been washed, when all treatments reduced entry rates by approximately half. N'Guessan *et al.* (2001) reported reductions in entry rates by resistant *An. gambiae* with standard Olyset nets (permethrin), using fresh nets (44% reduction) and aged nets that had been in the field for 3 years (20%). Agossa *et al.* (2015) also reported significant deterrent effects with Olyset nets, but, a later study in Benin found no evidence that standard Olyset net or Olyset Duo (permethrin + pyriproxyfen) impacted eave entry (Ngufor *et al.*, 2014).

With such variability in study design, treatments and treatment groups and the diversity of the target populations, it is not possible to draw conclusions about the deterrent properties of individual pyrethroids, let alone attempt to compare them. Moiroux *et al.* (2017) attempted to resolve some of these issues by running new trials to evaluate the most widely available ITNs. They termed deterrence at the eaves of a hut the 'Remote Effect', to distinguish it from close-range repellency closer to the bednet (see below). In their study, all unwashed ITNs provided significant personal protection over untreated nets but after 20 washes only three, the α-cypermethrin treated Interceptor, and the deltamethrin-treated PermaNet 2.0 and PermaNet 3.0 (deltamethrin + PBO), exerted a significant deterrent effect. As the authors state, very little is known of this important effect of pyrethroid-treated nets, and more research is needed into the durability of the effect after hanging the net, how it might differ between individual pyrethroid treatments and the true extent of its consequences in terms of public health.

5.3. Behavioural responses of pyrethroid-resistant mosquitoes

Vector populations can respond to IRS or ITN selection pressure with gross changes in behaviour such as shifts in time or location of biting, resting site preferences or host preference to avoid encountering the insecticide. However, less apparent behavioural changes that could confer partial resistance to pyrethroids may also occur, e.g. changes in sensitivity to repellent or irritant properties, modified blood feeding or resting behaviours. Potentially, less detectable changes in resistant mosquitoes might be associated with highly visible secondary consequences *e.g.*, thicker cuticle could result in changes in flight behaviour. Ultimately, any of these changes may significantly affect the efficacy of an ITN, in which case differences between pyrethroids should be considered if responding to pyrethroid resistance. In this review, research on *Aedes aegypti* has been excluded following recent evidence showing how the perception of repellents can be markedly different from those of anophelines (Afify and Potter, 2020; Hol, Lambrechts and Prakash, 2020). Contact-irritancy or excito-repellency is the response to irritation as an insecticide kicks in, resulting in the mosquito breaking contact and flying away (Kennedy, 1947; Muirhead-Thomson, 1960). It is a critical factor in ITN performance because the optimal dosage of insecticide must ensure the net delivers a lethal dose to every mosquito that lands on it before an irritation response is activated. The importance of the bednet's treatment in repelling or deterring mosquitoes may be indicated by the recent identification (at multiple locations across Africa where nets have been used widely for many years) of a previously unknown sensory appendage protein (SAP2) that appears to be essential for pyrethroid resistance (Ingham *et al.*, 2020). The

receptors are especially rich on the legs, typical of many chemoreceptors in mosquitoes (Yang *et al.*, 2020), but precisely what this means in terms of mosquito behaviour remains to be determined.

To date, there is no evidence that deltamethrin-treated nets can be detected by or can alter behaviour of susceptible *An. gambiae* or *An. arabiensis* prior to the first contact (Spitzen *et al.*, 2014; Parker *et al.*, 2015; Angarita-Jaimes *et al.*, 2016; Hughes, Foster, *et al.*, 2020). Deletre *et al.* (2019) reported altered responses to volatiles of DEET, natural repellents and permethrin associated with the *kdr* and ace1 mutations in pyrethroid resistant mosquitoes, where the effectiveness of each repellent increased or decreased depending on the mutation.

Post-contact, the lag time before irritation provokes flight is variable. Early studies with susceptible *An. gambiae* s.s. on human-baited netting reported resting times before disengagement of 3 and 6 minutes (Siegert, Walker and Miller, 2009) and 1.8 min and 3.2 min (Hodjati, Mousavi and Curtis, 2003) at Olyset and PermaNet 2.0, respectively. Cooperband and Allan (2009) found *An. quadrimaculatus* spent significantly longer times on deltamethrin than on bifenthrin or λ-cyhalothrin, but only after contact was made. Hughes, Foster, *et al.* (2020) found no evidence for deterrence, but recorded lag times between first net contact and the start of blood-feeding of 1 minute at untreated nets, and 2.5 and 3 minutes at both Olyset and PermaNet 2 nets.

Hughes, Foster, *et al.* (2020) described a sequence of behavioural events during blood-feeding with resistant (Tiassalé 13) and susceptible (Kisumu) forms of *An. gambiae*, at Olyset and Permanet 2.0 nets. The sequence was consistent regardless of net type or resistance status, but the duration of events varied with treatment. The most significant of these was a 50% reduction in the duration of blood feeding in both resistant and susceptible mosquitoes when they fed through either ITN, compared with untreated nets. Hauser, Thiévent and Koella (2019) reported a similar response in susceptible mosquitoes at Olyset Plus.

As discussed for bioassay studies in section 4A, obtaining a bloodmeal can lessen an insecticidal product's impact. Several studies have reported significantly higher survival rates in mosquitoes that blood feed during or immediately after exposure to insecticidal nets. First seen by Hossain and Curtis (1989) who reported 40% survival rates in *An. gambiae* fed through permethrin-dipped nets, the protective effect has also been recorded in resistant strains of *An. funestus* and *An. arabiensis* (Spillings *et al.*, 2008; Oliver and Brooke, 2014) and in wild populations in Burkina Faso (Hughes, Lissenden, *et al.*, 2020).

Conversely, failure to leave the net and/or feed rapidly after initial contact can impair the mosquito's ability or urge to seek or feed subsequently. Laboratory studies have shown significant reductions in host-seeking behaviour (i.e. probing through cage netting in response to a human host) at 1-hour post exposure to a PermaNet 2.0, of 80- 95% in resistant strains of *An. gambiae*, *An. arabiensis* and field-caught *An. funestus* (Glunt *et al.*, 2018). The effect was still significant at 24 hours in *An. arabiensis*. In similarly designed experiments, Thiévent *et al* (2019) reported impaired host seeking by *An. gambiae* for nearly 48 hours following sub-lethal exposure to Olyset nets; they also found that the presence of *Plasmodium* oocysts or sporozoites shortened the duration of inhibition of blood-seeking. The duration of the effect is important, because missing the next opportunity to successfully blood feed is likely to reduce that individual's reproductive output and its chance of successfully transmitting malaria. The timing also influences malaria transmission because of the thermal sensitivity of the early *Plasmodium* stages, such that vector competence is higher mosquitoes feeding in the evening (18:00) and lower for those feeding in the morning (06:00), relative to those feeding at midnight (00:00) (Suh *et al.*, 2020).

To date, only a few recent studies have documented how insecticide exposure affects the longevity of insecticide resistant survivors, despite its importance for disease transmission (Viana *et al.*, 2016; Tchakounte *et al.*, 2019; Hughes, Lissenden, *et al.*, 2020). Viana *et al.* (2016) observed reductions of up to 50% in mosquito lifespan in moderate and highly resistant strains of *An. gambiae* and modelled the data to estimate that this level of delayed mortality could reduce the malaria transmission potential of these populations by two thirds. In the population models, as the insecticide resistance intensity increased, the magnitude of the impacts on malaria transmission potential decreased, suggesting that the observed effects could be eroded by intensification of resistance. In semi-field populations exposed to PermaNet 2.0, Tchakounte *et al.* (2019) observed reductions in longevity of *An. gambiae* (F7) and *An. funestus* (F1) compared to unexposed mosquitoes. No evidence of delayed mortality following insecticidal net exposure was observed in a highly resistant wild populations from Burkina Faso (Hughes, Lissenden, *et al.*, 2020).

5.4. Limitations of existing WHO assays

Since the impact of any insecticide-based control method is determined ultimately by the mosquitoes' behavioural response at the point of delivery, the evidence for the efficacy of nets should derive from appropriate assays. The WHO assays currently used to evaluate insecticides for efficacy and durability, and to assess the impact of resistance on their performance, can no longer be considered fit for this

purpose. Bioassays such as the cone test record knockdown or mortality of non-blood fed adult female mosquitoes following unnatural levels of exposure to the active ingredient under highly artificial conditions. Measuring efficacy of insecticide solutions in such an environment will not predict how the eventual insecticidal net products or residual spray preparations used to deliver it to the target mosquito population will perform under field conditions, hampering informed deployment decisions. Similarly, monitoring for resistance with inadequate bioassays would not detect changes in behaviour that could compromise an ITN's performance. Within the confines of the CDC bottle bioassay or WHO tube test there is little scope to observe any behavioural response, and the only responses to exposure which are recorded are the scale, or speed, or knock down or kill.

Recent advances in understanding the behaviour of mosquitoes at the treated net interface have helped clarify the limitations of the WHO tests for efficacy testing. First, contact with the insecticide is 'forced', i.e. test mosquitoes are held in contact with the insecticide for a defined time, and any excito-repellency response that might enable a mosquito to escape and reduce contact is prevented. The three minute duration of forced exposure exceeds the time for most ITNs when irritation would provoke flight to break contact (less than 90 seconds, (Parker *et al.*, 2015; Hughes, Foster, *et al.*, 2020), within which nets must deliver a lethal dose. Secondly, it is the human occupant that makes an ITN so effective. Without a human attractant, mosquitoes do not orient to or contact netting at levels needed to receive a lethal dose of insecticide (Parker *et al.*, 2015). Moreover, without an attractant to oppose it, slightly repellent nets might appear very ineffective in small-scale tests like the WHO cone and tube tests, and at a larger scale, for example if investigating remote effects of any ITN treatment's deterrent properties at the eaves (Moiroux *et al.*, 2017). Yet the WHO cone test does not incorporate any attractant source, and the tunnel test uses a rabbit, not a natural host of malaria vectors.

The WHO test protocols record knockdown and mortality for up to 24-48 hours, using non-blood-fed 3-5-days-old adult mosquitoes, at their first exposure to insecticide. At this age, resistance is likely to be at its highest level, which increases the sensitivity of the test. While mass producing 3-5 day-old adult female mosquitoes for testing is also very convenient, in reality the mosquitoes transmitting malaria are likely to be two weeks post-emergence or older, with highly variable individual histories of blood feeding, insecticide contact, infection status and potentially also differences in resistance. These simple tests and protocols were intended for, and have been sufficient in, capturing the effects of fast-acting pyrethroids prior to the extent of resistance seen in Africa today. Their limitation for the future, is their inability to capture the full range of possible effects of sub-lethal insecticide doses, nor accommodate the detection of late impacts on longevity, reproductive output or development of

Plasmodium spp. Any effect of treatment that prevents or reduces the chances of a mosquito living long enough to become infective, or that impairs the likelihood of the mosquito biting at any time thereafter, potentially exerts a beneficial impact and should be quantified. Conversely, any resistance mechanism developed by the mosquito that reduces these effects could also be significant in determining whether a product will be effective against a given population.

Improving, augmenting or replacing tests as affordable, rapid and simple as the existing WHO tests with new assays that retain those properties as well as adding the ability to capture, distinguish and measure a range of outcomes without ambiguity, will be a challenge. These assays were designed as a litmus test for emergence of resistance, and as a means to evaluate fast acting pyrethroids, not to measure quantitative differences between mortality rates to inform product choices, and certainly not to draw any comparisons between the efficacy of different products or of levels of resistance to them. Carrasco *et al.*, (2019) have attempted to capture all anticipated behavioural events and other potential outcomes within a framework to guide classification and investigation. It is already apparent that any bioassay evaluating the efficacy of bednet treatments should include a host or a host mimic/surrogate. Ideally, the test should also permit discrimination between pre-contact repellency and post-contact irritancy, either of which could increase in importance as the range of chemical treatments increases. Other potential effects include duration of knockdown and behaviour on recovery, suppression and other impacts on blood-feeding ability or host-seeking behaviours and their duration. Ideally, resistance monitoring efforts should include methods to detect changes in those behaviours most likely to affect a product's performance.

5.4.1. New bioassay methods

As described above, a mosquito's behavioural response to an ITN is critical to its efficacy. It is therefore important to better understand whether the behavioural response to different pyrethroids is different, and so whether resistance (behavioural or otherwise) might diverge in response to selection pressure applied by different products. Benchtop systems that simulate natural exposure are being developed for routine use, which will help in this endeavour.

Sternberg, Waite and Thomas (2014) assembled a simple Mosquito Contamination Device (MCD) using two bottles: one filled with warm water to act as an attractant, the other the test arena for 10 mosquitoes (Figure 5.1C). Results from the MCD and cone tests were not dissimilar though slightly lower knockdown and 24-hour mortality rates in the MCD test might be result of lower levels of insecticide exposure to the test netting in the MCD test, where contact is not forced.

Grossman *et al.* (2020) divided a large cage with the netting being tested and studied the responses of mosquitoes on one side of the net as they responded to a human arm on the other side, thus mimicking natural exposure more accurately. The study showed that while a bloodmeal during exposure can mitigate the insecticide's impact, repeated exposure killed 97-98% by the time they would become sporozoite positive in nature.

The WHO Video Cone Test (VCT; Emery *et al.* (2019) retains the WHO setup, but adds considerable value by (1) incorporating a human host, and (2) recording mosquito activity during the 3-minute test using the video camera on a basic mobile phone (Fig 5.2D). Basic scan-sampling can be augmented by using ViCTA (Video Cone Test Analysis), a bespoke software to characterise the strength of contact irritation or excito-repellency by analysing mosquito activity recorded in the VCT (Fig 5.1F-G). The VCT-ViCTA package can be used in settings without access to any other tests.

In the Baited Box test, Hughes, Foster, *et al.* (2020) also used video to observe and quantify events in the detailed behavioural sequence of free-flying individual mosquitoes in a 10cm Perspex cube (Fig 5.1A,B). Mosquito activity is recorded as the mosquito arrives at and lands on a human-baited bed net, where blood-feeding on the human host through the bednet can be permitted or prevented. The Baited Box test was designed as a partner to the room-scale flight tracking system (Angarita-Jaimes *et al.*, 2016) to provide details of the closing stages of mosquito-net interaction that are not captured in the larger-scale assay.

5.4.2. Large-scale systems

Several permanent or full-scale bioassay systems, originally developed as experimental tools are being used to characterise a broad range of properties of different ITNs and to investigate the behaviour of pyrethroid resistant vector populations. Both systems are unlikely to be operated by any more than a few key laboratories or testing centres, ideally certified to monitor the performance and durability of the growing numbers of net brands, and of the resistance status of target vector populations.

The Ifakara Ambient Chamber Test (I-ACT) is a testing facility located at Bagamoyo, Tanzania (Massue *et al.*, 2019). Housed in a permanent structure (Fig 5.2A), this 50m long unit houses 10 independent semi-field units for testing and quantifying the functional efficacy of nets, including durability studies on worn and torn nets taken from the field. Each unit (Fig 5.2B) is independently temperature

controlled and designed to accommodate a human-occupied bednet and the net's performance is determined from the effects on 30 released mosquitoes over a 12-hour period.

A room-scale video-tracking system was designed as a semi-portable system suitable for use under field conditions (Angarita-Jaimes *et al.*, 2016). This 2D tracking system was used to define behaviour of *An. gambiae* s.s. at a bednet in the laboratory and later with *An. arabiensis* in the field in Tanzania. Use of this system quantified the importance of the ITN roof as the primary point of contact for *Anopheles* sp. and *Culex quinquefasciatus* and revealed the distribution of spatial activity around a net that led to the development of the barrier bednet (Murray *et al.*, 2020). Tracking systems are now being used by a growing number of research laboratories and almost certain to provide key evidence to guide the selection of ITN treatments.

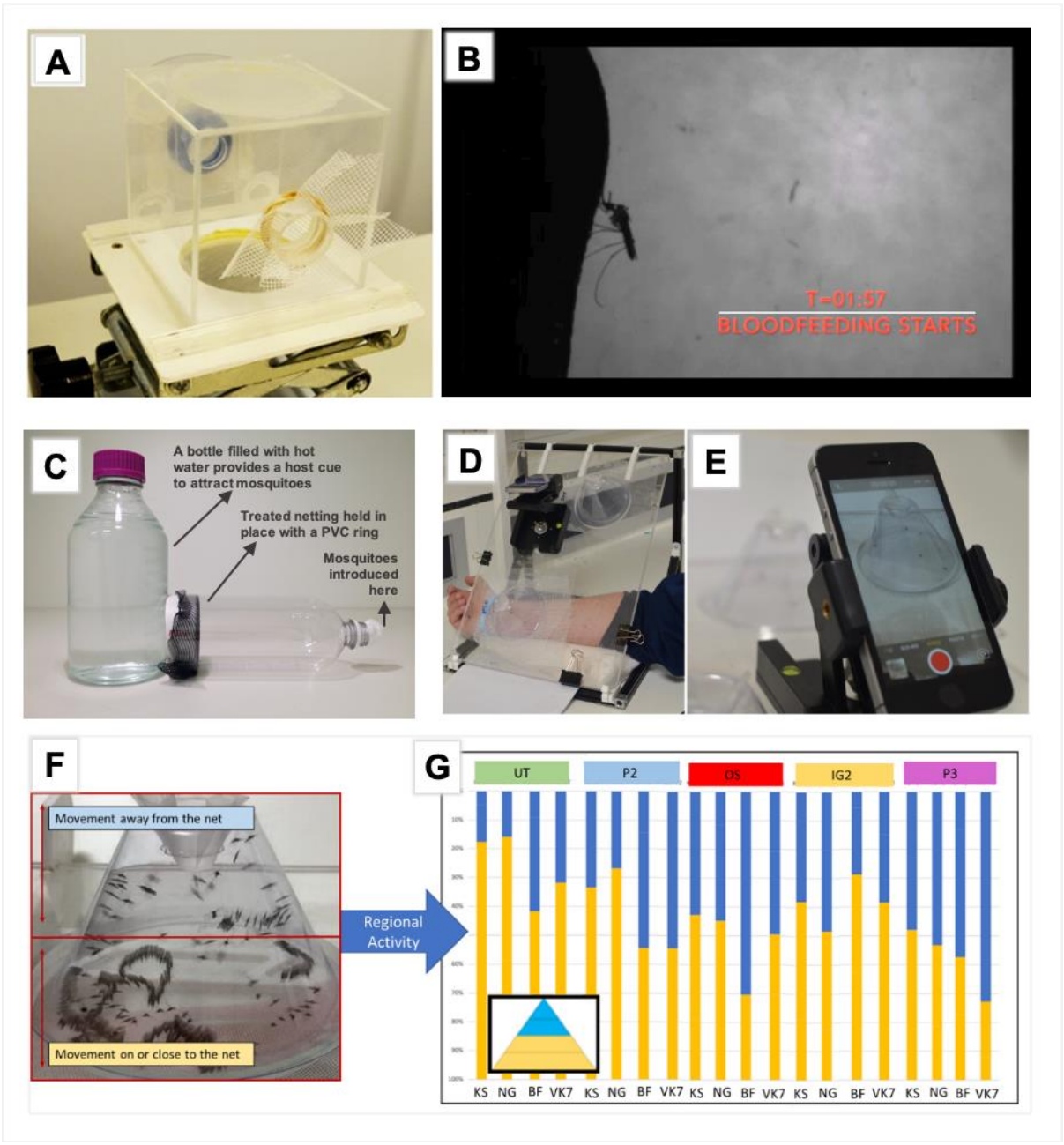


Figure 5.1. Bench-top bioassays: (A) The Baited box test arena, with the test netting at the front and mosquito entry port at the rear; (B) screen capture from a baited box assay when feeding is permitted); (C) The MCD bottle bioassay test apparatus; (D) the WHO Video Cone Test with the operator's arm and mobile phone mounted on the purpose-built rig and (E), the recorded field of view. (F) ViCTA (Video Cone Test Analysis) creates a composite image of all mosquito positions recorded at 0.1sec intervals over the total 3 minutes and captures movement; (G) proportions of activity in upper or lower cone regions are indicative of strength of irritation/excito-repellency, shown here for untreated netting and for Permanet 2.0, Olyset, Interceptor G2 and Permanet 3 ITNs, tested against 4 strains of *An. gambiae*. Images from Hughes, Foster *et al.* (2020)(A,B); Sternberg, Waite and Thomas (2014)(C); D-G.

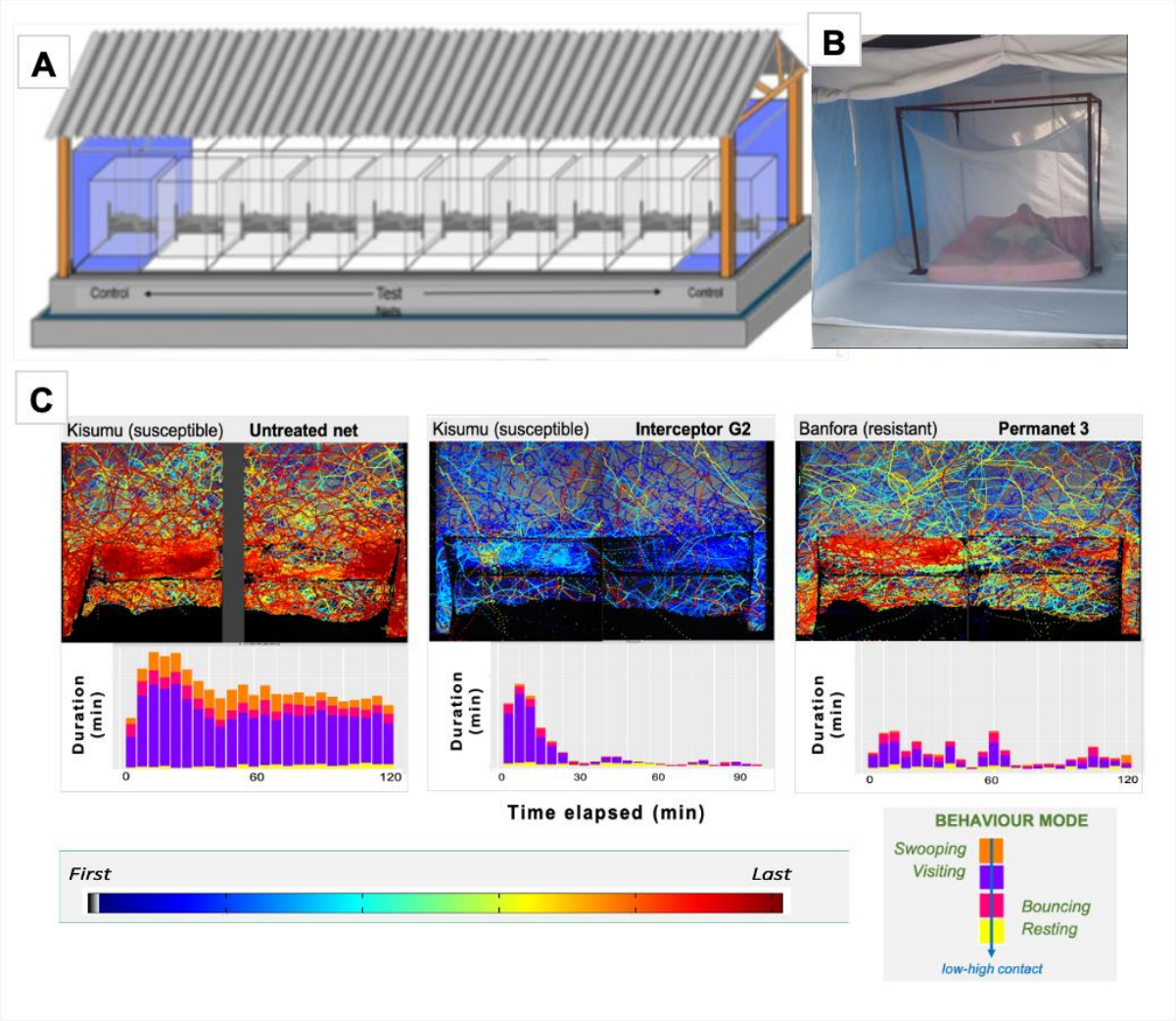


Figure 5.2. Full-scale bioassay and testing facilities: (A) The Ifakara Ambient Chamber Test or I-ACT is a testing facility located at Bagamoyo, Tanzania. This 50m long unit comprises 10 independent temperature-controlled test units, each of which (B), is designed to accommodate a human-occupied bednet; the net's performance is determined from the effects on 30 released mosquitoes over a 12hour period. (C) Examples of output from the Room Scale Video Tracking System: the image shows every flight path seen in a 120min video of 25 *An. gambiae* females activity around a human-occupied bednet as labelled; Tracks are colour-coded according to time they first appeared in the field of view as shown in the key, from blue (Start) to red (end) . The graph shows the total activity over 120mins in each behaviour mode (Parker *et al.*, 2015)

5.5. Conclusions

- The overall response of pyrethroid resistant mosquitoes to an ITN may comprise alterations in multiple behaviours and any differences in responses to individual pyrethroids provide an opportunity for the efficacy of pyrethroids to diverge.
- Current resistance monitoring bioassays are unable to detect differences between responses to different insecticides or differences between susceptible and resistant mosquitoes, yet the results of these bioassays may be confounded by such changes in behaviour.
- A better understanding of behavioural responses and how they differ between susceptible and resistant mosquitoes should inform the deployment of the most effective products. This will require the development and adoption of new bioassays. Methodologies to collect more valuable data about the behavioural response of *Anopheles* mosquitoes under more relevant conditions, including large scale testing arenas, are under development.

6. Overall findings & conclusions

6.1. The evidence for divergent resistance to different pyrethroids

- Evidence from discriminating dose bioassays, resistance intensity bioassays and experimental hut trials all indicate on average slightly higher mortality to Type II pyrethroids, compared to Type I, in wild mosquito populations. This difference is unlikely to have a substantial public health impact, especially when intrinsic differences between ITNs, such as the surface bioavailability of insecticide, are considered. It is consistent with the finding that the discriminating dose for permethrin and deltamethrin may induce slightly different levels of mortality
- Together molecular studies of metabolic resistance and studies of the resistance in mosquito populations (including analyses of intensity data, of diagnostic dose bioassay data from populations that have been tested with multiple pyrethroids, and of spatio-temporal trends) provide evidence that there is strong cross-resistance, particularly between permethrin and deltamethrin.
- P450s-SAR (structure-activity relationship) findings conclude that the more commonly used pyrethroids are the most vulnerable insecticide for metabolic attack (Cytochrome P450s) while bifenthrin, λ-cyhalothrin and α-cypermethrin are less vulnerable for metabolic attack.
- Bioassay data from *Aedes aegypti* and *Anopheles sinensis* suggest that bifenthrin may show relatively low cross-resistance with other more commonly used pyrethroids. Bifenthrin has not been widely used in malaria control in Africa and no diagnostic dose has been defined but its potential use in malaria vector control warrants further investigation.
- Trends in mortality over time do not suggest divergence in resistance between deltamethrin and the other pyrethroids has occurred in laboratory strains selected with deltamethrin for up to 6 years.
- In field populations, variability in discriminating dose and dose-response assay mortality is high. This variability is predominantly at a local geographical scale indicating that if there were a difference between Type I and II pyrethroids it will be so local and beneath the size of the region insecticidal nets are currently allocated.
- There is good evidence that the mortalities from exposure to deltamethrin, permethrin, α-cypermethrin and λ-cyhalothrin are strongly correlated across *An. gambiae* s.l. communities and environments. These correlations were also seen for deltamethrin, permethrin and λ-

cyhalothrin (α-cypermethrin was not tested) in the *An. funestus* subgroup and in all three of the main malaria vectors within the *An. gambiae* complex.

- There is evidence that resistance to etofenprox could diverge from resistance to the more commonly used pyrethroids, however, without further investigation, the potential vulnerability to metabolic attack by P450s shown by molecular studies might caution against an operational switch.

6.2. The suitability of current testing methods to monitor insecticide resistance and make deployment decisions

- Deployment decisions are being made based on the discriminating dose and resistance intensity bioassay results, but it is not clear how well differential mortality in WHO tube or CDC bioassays predict how well an ITN treated with one or other pyrethroid will perform in a specific site. Bioavailability may play an important role in relative efficacy of different ITNs, and testing for relative performance of different nets against field populations might better inform deployment decisions, alongside or in place of conventional bioassays using the insecticide alone applied to a filter paper or bottle.
- Given the limited products available for vector control, and narrow collection of available chemistries, programmes must make deployment decisions based on the data which can realistically be collected. The current monitoring system for insecticide resistance is imperfect but should be adapted to make better use of the available resources.
- In general, following exposure of characterised lab strains in WHO tube bioassays under controlled conditions intra-strain mortality to permethrin, deltamethrin and α-cypermethrin was similar. However, in intermediately resistant strains some divergence in mortality rates was observed. The level of variability in observations of mortality between tubes (measured using standard deviation) was also greater in these moderately resistant strains.
- When comparing resistant lab strains, both mortality level and variability in mortality is greater in the CDC bottle bioassay compared to the WHO tube test.
- Diagnostic dose assays are poor tools for quantitative analysis of resistance levels where resistance is established, producing the most variability in results in laboratory colonies and field populations where resistance is moderate and mortality is intermediate, which is now likely to be the case for all or most pyrethroids in most populations of African malaria vectors.
- For comparisons across insecticides intensity assays suffer from the same problem as the diagnostic doses on which they depend – an apparent lack of parity across insecticides.

Quantitative dose-response assays, which do not depend on a diagnostic dose are the preferred method for robust comparisons among products.

- All bioassays are vulnerable to very strong effects of humidity and temperature, in addition to other environmental effects more easily standardized by the user. It is crucial that better reporting of deviations from optimal conditions are reported, along with improved provision of sampling details, to understand the likely population-representativeness of collections.

7. Recommendations

7.1. Making deployment decisions about different pyrethroids on the basis of resistance monitoring data

- Based on the available compelling evidence from both molecular studies of metabolic resistance and in vivo studies of resistance in mosquito populations, it is inadvisable to switch between the pyrethroids that are in common use (deltamethrin, permethrin, α-cypermethrin and λ-cyhalothrin).
- Improved evidence for whether resistance to less common pyrethroids such as bifenthrin is divergent from commonly used pyrethroids, is needed before a switch could be recommended.
- Systematic quantitative structure activity relationship (QSAR) using LC_{50} values calculated from dose-response data is are required to determine the bio-efficacy of the full range of pyrethroids including bifenthrin, etofenprox, α-cypermethrin, cyfluthrin, lambda-cyhalothrin, deltamethrin and permethrin against wild populations of malaria vectors which are resistant to deltamethrin and/or permethrin. This will allow a comprehensive understanding of the impact of resistance mechanism(s) on the bio efficacy of each molecule.
- Current tests lack the sensitivity to detect operationally significant differences in resistance to different pyrethroids at a single location. A difference found at a single time and place may be a true difference or the result of measurement error in the assay. Any decision to switch between pyrethroids needs to be based on tests conducted at multiple sites across the intervention target area. This approach also addresses the variation in vector populations across the target area.
- Ideally the vectors transmitting malaria should be tested and results should be obtained for each species biting humans within each target area. If this is not feasible, the evidence indicates that patterns of resistance within a species complex within an area are broadly consistent whereas there are key differences between *An. gambiae* s.l. and the *An. funestus* subgroup. Where both are present, it is vital that both are tested.
- Historical results from tests at a single site using different pyrethroids could be combined to provide a more robust estimate of pyrethroid resistance to inform decisions on the deployment of, for example, PBO-treated nets.

7.2. Improving the collection and use of resistance monitoring data and further investigation to better inform deployment decisions

- In order to better understand the suitability of the discriminating doses for permethrin and deltamethrin it would be valuable to compare dose response data from a range of susceptible *Anopheles* strains. Better calibrated DDs could then be established, and the relative potency of the pyrethroids confirmed. A meta-analysis of the literature to establish if such data exists is recommended, the results of which can then be compared to the current recommended diagnostic doses. If existing data is limited, a small-scale trial to confirm the LD values for permethrin and deltamethrin to several *Anopheles* species, ideally using multiple strains of each, should be conducted.
- Bioassays for insecticide resistance monitoring rely on wild collection of often limited numbers of mosquitoes and should therefore focus on the insecticide class/es of primary interest for current or future operational decision making.
- Although demanding in terms of mosquito requirements for testing, dose-response bioassays (independent of diagnostic doses) remain the preferred method for comparative assessment of insecticide resistance among insecticides. Ideally, and to facilitate comparisons across studies, they should also involve a standard fully-susceptible laboratory strain for calculation of resistance ratios.
- In addition to avoiding testing in uncontrolled conditions where possible, improved reporting of rearing and testing conditions is crucial to allow consideration of possible biases, along with more details of sampling protocols to assess likelihood of sampling populations representatively.
- If limited numbers of mosquitoes are available for testing, the use of PBO bioassays are not recommended for the following reasons. The issues of data noise are magnified because the end result is the difference between two bioassays. Further, most tests reveal a positive result if the WHO criteria for presence - an increase of more than 10% mortality - is used, so the evidence will almost always support deployment of PBO-treated nets. Finally, these tests cannot provide a more nuanced quantitative assessment of the level of P450-mediated resistance.
- In general, interpretation of PBO bioassay test results is challenging, with some authors apparently seeking a 10% change, but most either a full return to susceptibility or evidence of a statistically significant increase in mortality compared to the insecticide only exposure. In any of these cases, operational efficacy is difficult to predict confidently. A more practical and operationally relevant alternative might be an increase in testing products directly using cone tests, or where practical video cone test assays.
- No evidence has been found for differential efficacy of PBO nets on the basis of which pyrethroid they include. In order to better understand the possible operational implications for recommendations on the use of PBO nets in areas of pyrethroid resistance, further investigation

is needed to elucidate the biological impact of PBO on P450s, and the molecular basis for any differential efficacy of PBO in synergising different pyrethroids.

- Potential differences in behavioural responses to the different pyrethroids will not be captured by conventional resistance monitoring bioassays, and indeed results may be confounded by different levels of excito-repellency or avoidance behaviour. More research is needed to develop appropriate bioassays for the full range of behaviours that can influenced the efficacy of pyrethroids against resistant mosquitoes.

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