Impact of pyrethroid resistance on operational malaria control in Malawi

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The impact of insecticide resistance on insect-borne disease programs is difficult to quantify. The possibility of eliminating malaria in high-transmission settings is heavily dependent on effective vector control reducing disease transmission rates. Pyrethroids are the dominant insecticides used for malaria control, with few options for their replacement. Their failure will adversely affect our ability to control malaria. Pyrethroid resistance has been selected in Malawi over the last 3 y in the two major malaria vectors *Anopheles gambiae* and *Anopheles funestus*, with a higher frequency of resistance in the latter. The resistance in *An. funestus* is metabolically based and involves the up-regulation of two duplicated P450s. The same genes confer resistance in Mozambican *An. funestus*, although the levels of up-regulation differ. The selection of resistance over 3 y has not increased malaria transmission, as judged by annual point prevalence surveys in 1- to 4-y-old children. This is true in areas with long-lasting insecticide-treated nets (LLINs) alone or LLINs plus pyrethroid-based insecticide residual spraying (IRS). However, in districts where IRS was scaled up, it did not produce the expected decrease in malaria prevalence. As resistance increases in frequency from this low initial level, there is the potential for vector population numbers to increase with a concomitant negative impact on control efficacy. This should be monitored carefully as part of the operational activities in country.

The push for malaria elimination and eventual eradication will be heavily dependent on our ability to reduce disease transmission. A recent editorial suggests that we have the tools to take on this challenge in African malaria heartlands (1). This is predicated on ensuring that vector control prevention and drug treatment tools are fully deployed, reaching every person at risk. There will need to be improved delivery of these tools and better clinical management of malaria cases. In highly endemic areas our ability to reduce malaria transmission will be dependent on vector control, before the focus can shift to killing the parasite in infected people. Two forms of vector control, indoor residual spraying (IRS) and the distribution of long-lasting insecticide-treated nets (LLINs) have been demonstrated to reduce transmission when properly deployed against insecticide susceptible mosquito populations. The use of both interventions has dramatically increased since 2000 in many malaria endemic countries, with increased donor funding to attain the Roll Back Malaria targets and support the malaria elimination agenda (2).

IRS and LLINs function by reducing the female mosquito daily survival rate and human biting frequency. Pyrethroids are the only insecticides recommended for use on LLINs, and only four chemical classes of insecticides that attack two target sites are available for IRS, and again pyrethroids dominate the IRS market. Resistance to pyrethroids has been selected in *Anopheles gambiae* and *Anopheles funestus*, the major African malaria vectors, although the frequency and level (fold) resistance conferred can vary dramatically. The impact of this resistance on the ability of either control intervention to reduce disease transmission is poorly understood, and current monitoring and evaluation practices are not sufficiently robust to assess this unless catastrophic failures occur. The perceived threat of pyrethroid resistance is now sufficiently high for the World Health Organization (WHO) to convene an international multidonor effort to counteract this.

Operationally significant pyrethroid resistance has the potential to limit effective malaria control, owing to the small number of alternative public health insecticides. Pyrethroid resistance in malaria vectors has increased dramatically over the last decade (3, 4), particularly in Africa, where the bulk of malaria-related mortality occurs. Typically resistance is monitored by bioassays, for which the WHO has defined a diagnostic dosage for each insecticide that kills susceptible anopheline mosquitoes (5). Mosquitoes surviving the diagnostic dosage are an indication that resistance has been selected and that an operational problem may be developing, but bioassays alone do not signify control failure.

Little operational monitoring of the underlying mechanisms of resistance occurs. Two mechanisms are predominantly responsible for insecticide resistance: changes in the insecticide target site, reducing binding of the insecticide, and increases in the rate at which the insecticide is metabolized (6). Information on the resistance mechanisms is more predictive than bioassays, providing information on the level of resistance and potential cross-resistance between insecticides. For example, two common mutations in the sodium channel convey low-level resistance to pyrethroids and higher-level resistance to dichlorodiphenyltrichloroethane (DDT) in *An. gambiae* (7, 8), whereas a cytochrome P450-based metabolic regulatory mechanism conveys very high-level pyrethroid and low-level carbamate resistance in *An. funestus* (9).

Vector control interventions are being rapidly scaled up in Malawi, where malaria is highly endemic. Malaria accounts for 34% of all outpatient hospital visits and is the main cause of hospital admissions in children aged <5 y (10). Before 2007 sporadic WHO bioassays were undertaken, which indicated that the two major malaria vectors, *An. gambiae* and *An. funestus*, remained fully susceptible to pyrethroids. In 2007 pyrethroid-impregnated LLINs were distributed through antenatal and under-5 clinics at district and central hospitals countrywide. The numbers distributed were sufficient to achieve the Roll Back Malaria targets of 80% of pregnant women and children aged <5 y sleeping under a treated net. In 2008, a pilot study of IRS with the pyrethroid lambda cyhalothrin (ICON, Syngenta) was initiated in Nkhota Khota District, supported by the President's Malaria Initiative (PMI). The initial program targeted 26,950 houses, and was expanded to 74,772 houses in 2009. Approximately 4 million LLINs were procured and ~2 million distributed during this time. In

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Data deposition: The sequences reported in this paper has been deposited in the GenBank database (accession nos. JN815112, JN815113, JN815114, JN815115, and JN815116).

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2010 the PMI-supported IRS was expanded to cover the whole of Nkohta Khota district, and the Malawian Ministry of Health supported IRS in a further six districts.

A series of sentinel sites were established during this period to track the effect of this rapid increase in insecticide selection pressure on the local vectors and assess any impact on malaria transmission. This was particularly pertinent owing to the high levels of pyrethroid resistance reported in the southern part of neighboring Mozambique in *An. funestus*, which had prompted a switch from pyrethroids to carbamates or DDT for IRS in the Lubombo Spatial Development Initiative area of Mozambique (11, 12).

Results and Discussion

Few operational reports exist to compare the impact of insecticide resistance directly on epidemiological outcomes of disease, owing to the number of confounding factors. Most studies have been undertaken with LLINs, often in experimental hut settings, and use entomological outcomes to assess the effect of insecticide resistance on mosquito biting rates, blood feeding rates, or insect mortality (13–15). Even these data are difficult to interpret, because of variation in experimental design and inconsistent and often poor-quality insecticide resistance monitoring of the vector population.

Recently Malawi has begun to scale up its malaria control program with LLIN and IRS initiatives. By monitoring these activities in 12 sentinel sites as they are scaled up, and simultaneously assessing pyrethroid resistance levels in the malaria vectors over the period that resistance was initially selected, this study provides an initial assessment of the impact of known pyrethroid resistance mechanisms on an operational malaria control program.

Insecticide Resistance Detection. An early indicator of the selection of resistance in South Africa was the presence of *An. funestus* resting inside sprayed houses (16). Over the course of 2009–2010 in Malawi it became easier to find *An. funestus* in substantial numbers resting inside recently insecticide-sprayed houses. A total of 460 resting female *Anopheles* mosquitoes were collected inside houses from 12 localities between April 2009 and April 2010. Of these, 340 were morphologically identified as belonging to the *An. funestus* group and 120 to the *An. gambiae* complex.

WHO bioassays were carried out on 2,579 F_1 *Anopheles* offspring from 12 sentinel sites (Fig. 1) and on a further 1,115 *An. funestus* from the Chikwawa site using randomly mixed F_1 individuals. *An. funestus* from the sentinel sites were resistant to the carbamate, bendiocarb (24–45% survival), and three pyrethroids (8–66% survival) (Table 1). There was no evidence of organophosphate or DDT resistance. The *An. funestus* population from Chikwawa had 53% survival of females and 42% survival of males after exposure to the discriminating dosage for permethrin. Marginally higher levels of resistance (58% female and 48% male survival) were recorded for deltamethrin. The Chikwawa population was also resistant to bendiocarb and had low levels of resistance to DDT, but was fully susceptible to dieldrin.

An. gambiae had lower levels of resistance than *An. funestus*, but permethrin resistance was detected at all sentinel sites tested, and at one none-IRS site (Nkhwazi) there was also resistance to lambda cyhalothrin. The same field populations of both species tested in 2007 was fully susceptible (100% mortality) to all insecticides (Table 1).

In contrast to DDT resistance selection in several African locations during the first malaria eradication campaign, where *An. gambiae* rapidly became resistant whereas *An. funestus* populations were often eliminated, in Malawi *An. funestus* has been selected for pyrethroid resistance more rapidly than *An. gambiae.* Hence, where both vectors are present it should not be assumed that *An. funestus* will be easily controlled by the introduction of pyrethroids.

Biochemical Assays. Efforts to elucidate the underlying mechanisms of resistance focused on *An. funestus*, for which increased

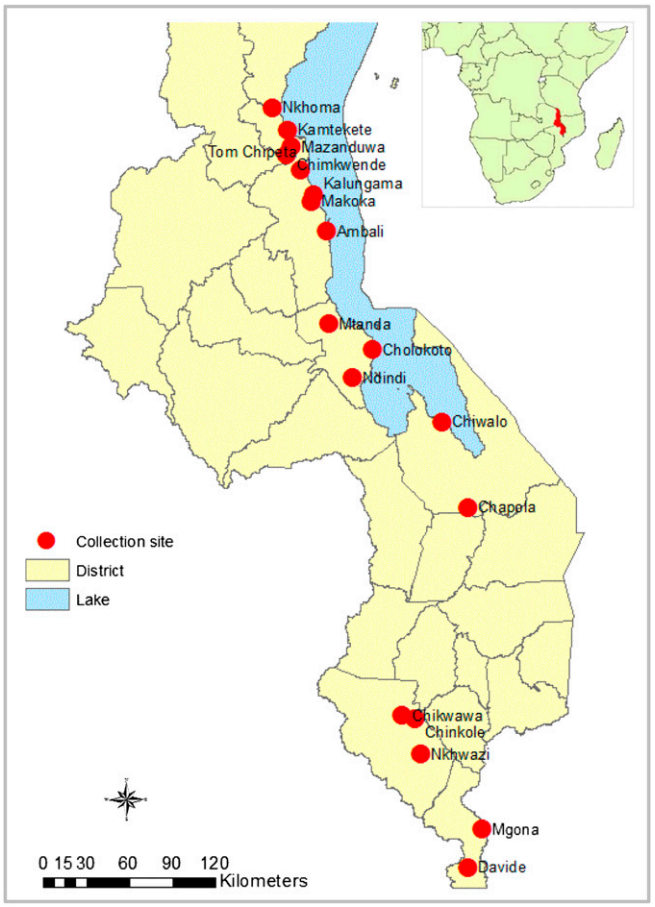

Fig. 1. Map of Malawi showing the localization of the different collection sites.

numbers resting on sprayed surfaces, higher resistance levels, and greater numbers suggested there might be the greatest impact on disease transmission. Many of the enzyme systems involved in insecticide resistance are controlled by common regulatory cascades, hence a change in a single regulator can affect the production of multiple enzymes from different classes, making it impossible from simple biochemical assays to extrapolate to the number of resistance genes that have been selected. These assays do, however, indicate a shift in metabolic activity that can then be further characterized.

The biochemical assays indicated that the Malawian *An. funestus* population had increased metabolic activity of several enzyme families compared with a standard susceptible *An. gambiae* laboratory population, which has metabolic activity levels consistent with a raft of different insecticide susceptible Anopheles mosquito species. Elevated esterases can produce organophosphate, carbamate, or pyrethroid resistance. Resistance to the first two insecticide classes has been routinely associated with activity that can be detected with the substrates α- and β-naphthyl acetate. There was a significant (1.82-fold) increase in esterase activity with the substrate p-nitrophenyl acetate (pNPA) in the Chikwawa population compared with the Kisumu susceptible strain ($P = 0.027$) (Fig. 2*A*). In contrast no increase was seen with either of the naphthyl acetate substrates.

A significant increase in the level of GST activity was observed in Chikwawa, with mean chlorodinitrobenzene levels 2.05-fold higher than Kisumu ($P < 0.0084$) (Fig. 2*C*). GSTs break down DDT and catalyze secondary metabolic detoxification pathways for many other insecticides.

The greatest increase was seen with monooxygenases: a 2.35-fold increase in total monooxygenases was detected in

Table 1. WHO susceptibility test results on 2- to 5-d-old F_1 *An. gambiae* and *An. funestus*

	Insecticide											
	Bendiocarb (0.01%)		Deltamethrin 0.05%		Lambda-cyhalothrin 0.05%		Permethrin 0.75%		Pirimiphos methyl (0.9%)		DDT 4%	
Locality	M	*n*	M	*n*	M	*n*	M	*n*	M	*n*	M	*n*
An. funestus												
Chapola							91.7	24				
Chikwawa females	60	80	42.3	104			47.2	182			87.8	74
Chikwawa males	55	98	52	100			58.1	227			100	50
Chinkole			68.5	127	60	125						
Chiwaro							78.9	19				
Kalungama	76	54	51.2	82	34.3	140	67.4	46				
Mwazanduwa	69	110	39.4	132	35.3	156	75.5	155			99	102
Nkhwazi					100	26						
An. gambiae												
Chinkole			100	31			100	16				
Davide							82	62				
Mgona							94	51				
Mtanda							98.9	91				
Ndini							100	34				
Nkhwazi	100	28	100	80	65.5	84	92.9	184	100	23		

M, percentage mortality; *n*, number tested.

the Chikwawa population (Table 2). The biochemical data are consistent with one or more metabolic resistance mechanisms having been selected in the Malawian *An. funestus*, which warranted further molecular characterization.

Acetycholinesterase (AChE) inhibition rates with the carbamate propoxur were similar in the resistant and susceptible strains (Fig. 2*B*). The lack of an altered target site resistance mechanism for organophosphates or carbamates was further confirmed by sequencing and hence can be discounted, suggesting that the bendiocarb and pyrethroid resistances detected by bioassay have a metabolic basis.

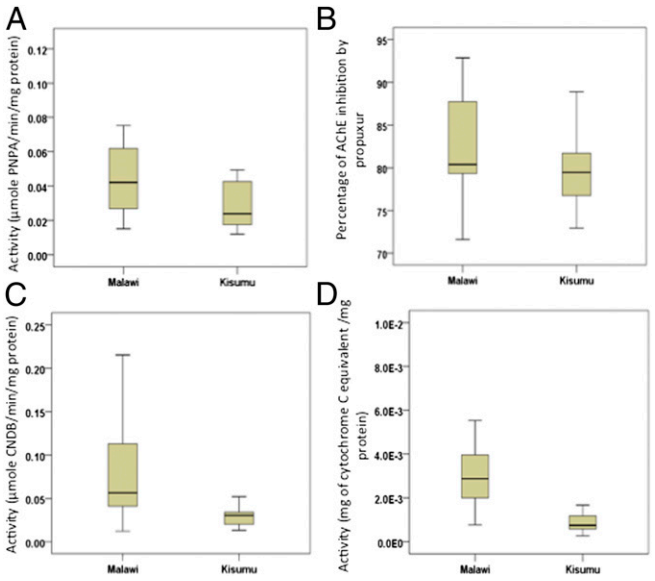

Fig. 2. Box plots of results from biochemical assays. The median activity of the *An. funestus* population of Chikwawa compared with the *An. gambiae* Kisumu reference strain is shown by a horizontal bar; the box denotes the upper and lower quartiles. The vertical lines show the full range of the data set. (*A*) Range of esterase activity with the substrate p-nitrophenyl acetate; (*B*) acetylcholinesterase inhibition ranges. (*C*) Range of GST activity. (*D*) Estimated levels of cytochrome P450s (representing monooxygenase activity).

Transcription Profiling of Candidate P450 Genes. The increase in monooxygenase levels seen by biochemical assay prompted the assessment of expression levels of the two P450 genes (*CYP6P9a* and *CYP6P9b*) that are primarily responsible for pyrethroid resistance in *An. funestus* in a laboratory strain and from field-collected insects from neighboring Mozambique (17). Expression levels were assessed in individuals that were known to be permethrin or bendiocarb resistant, and these were compared with susceptible or control mosquitoes from the same population that had not been exposed to insecticide. Quantitative PCR showed that *CYP6P9a* was consistently and significantly more over-expressed than *CYP6P9b* ($P < 0.01$). The increased expression was apparent in permethrin resistant (1.65-fold), bendiocarb resistant (3.15-fold), and control (2.15-fold) individuals compared with susceptibles (Fig. 3*A*). This expression pattern differs from that observed in pyrethroid resistant Mozambican *An. funestus*, in which both P450s have a comparable level of expression, with *CYP6P9b* being slightly but nonsignificantly higher. The lower fold change difference between both genes in permethrin resistant insects may indicate that both enzymes are involved in pyrethroid metabolism, whereas only *CYP6P9a* may play a role in bendiocarb metabolism.

The expression profile of *CYP6P9a* shows that this gene had significantly higher expression in permethrin resistant mosquitoes than in bendiocarb resistant ones (1.69-fold change; $P < 0.001$) (Fig. 3*B*). It was also more highly expressed in permethrin resistant than in control mosquitoes (not exposed to insecticide), although the difference was not significant (1.27-fold change). *CYP6P9a* expression in control insects was higher than in bendiocarb resistant mosquitoes, although the difference was not significant (1.33-fold change).

CYP6P9b was significantly over expressed (3.24 fold, $P < 0.001$) in permethrin resistant compared with bendiocarb resistant ones, compared with 1.68 for *CYP6P9a* (Fig. 3*C*). The expression of *CYP6P9b* in permethrin resistant mosquitoes was significantly higher than in bendiocarb resistant ones (1.95-fold, $P < 0.05$). Both *CYP6P9a* and *CYP6P9b* were significantly overexpressed in all of the samples tested compared with the susceptible strain FANG (Fig. 3*B*, $P < 0.001$). The highest fold change for both enzymes was observed in permethrin resistant compared with

Table 2. Comparisons of average values for a range of biochemical assays between F_1 adult progeny *An. funestus* from Chikwawa populations and the *An. gambiae* Kisumu insecticide susceptible reference strain

Assay	Mean (SD) Chikwawa	Mean (SD) Kisumu	Fold change	*P* value
pNPA	0.051 (0.037–0.063)	0.028 (0.015–0.042)	1.82	0.027
α-Naphthyl acetate	1.34 10^{-05} (5.2 10^{-6}–2.1 10^{-5})	1.58 10^{-05} (9.4 10^{-6}–2.2 10^{-5})		0.64
β-Naphthyl acetate	8.12 10^{-05} (2.2 10^{-5}–1.4 10^{-4})	6.47 10^{-05} (1.9 10^{-5}–1.1 10^{-4})		0.66
P450	0.0033 (2.4 10^{-3}–4.1 10^{-3})	0.0014 (7.2 10^{-4}–2.05 10^{-3})	2.35	0.0012
GST	0.082 (0.058–0.11)	0.04 (0.021–0.059)	2.05	0.0084
AChE	82.6 (79.5–85.4)	79.9 (77.0–82.7)		0.18

FANG (8.9- and 10.5-fold change, respectively), followed by nonexposed/FANG comparison with 7.1- and 6.3-fold change, respectively, supporting the potential role of these two genes in pyrethroid resistance in Chikwawa.

Mutations of the Sodium Channel Gene. Resistance due to changes in the target site of pyrethroids and DDT can be directly assessed by sequencing the relevant section of the target site. A 994-bp fragment of the voltage-gated sodium channel (VGSC) gene was sequenced. A total of 10 Chikwawa *An. funestus* specimens were sequenced (5 permethrin resistant and 5 susceptible mosquitoes). A 917-bp sequence from this fragment previously aligned in pyrethroid resistant Ugandan mosquitoes (18) was aligned for all individuals. Table 3 shows that there were 10 polymorphic sites, with four transitions and six transversions. Ten polymorphic sites were recorded in the resistant samples and only one in the susceptibles. No polymorphic sites were observed in exon 20 containing the 1014 codon usually associated with *kdr*-type pyrethroid resistance in other Anopheles. Only five haplotypes were observed in total (one for the susceptibles and three for resistants, with one in common); in contrast, 18 haplotypes were found in the same fragment from Ugandan *An. funestus*. The haplotype sequences have been submitted to GenBank (accession nos. JN815112–JN815116).

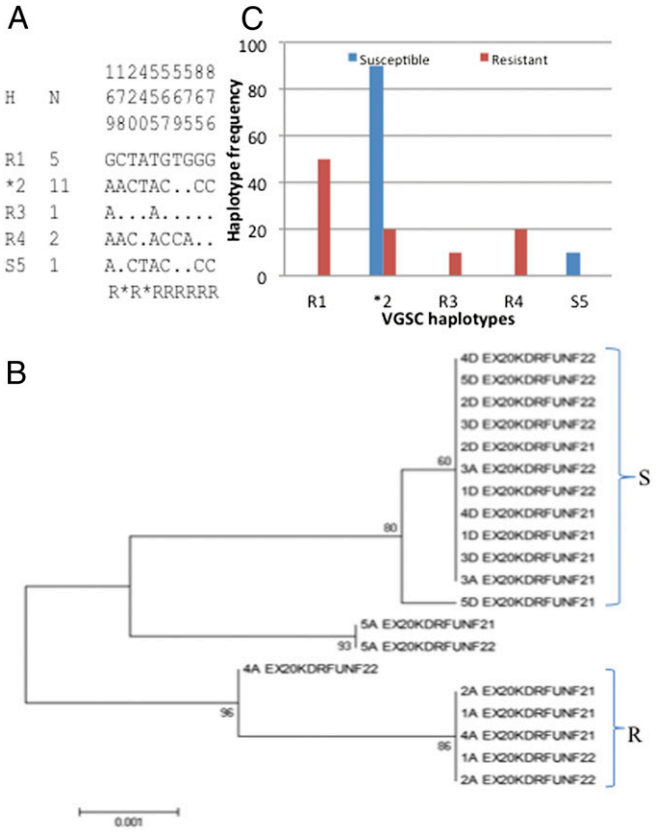

Fig. 3. (*A*) Schematic representation of haplotypes (H) of the 917-bp portion of the VGSC observed in the Chikwawa population. Only polymorphic sites are shown, and these are numbered from the beginning of the 917-bp sequence. Dots mean identity with the first sequence. A number has been given to each haplotype preceded by the letter R or S if it is unique to the resistant or susceptible sample, respectively. In case of a shared haplotype, the number is preceded by an asterisk. The column (N) indicates the number of individuals sharing the haplotype. Below the list of haplotypes, R or S indicates the positions that are polymorphic in the resistant or susceptible mosquitoes, respectively, whereas an asterisk marks a position polymorphic in both phenotypes. (*B*) Neighbor-joining tree of the VGSC haplotypes from Chikwawa. (*C*) Distribution of the sodium channel gene haplotypes between susceptible and resistant mosquitoes from Chikwawa.

The correlation between patterns of polymorphism and resistant phenotype was further confirmed by the neighbor-joining tree showing specific clades for resistant and susceptible individuals (Fig. 4*B*). This correlation suggests that although a 1014 mutation was not identified in Malawian samples, changes in the VGSC gene may still confer permethrin resistance. To confirm that changes in 1014 were not present, pyrosequencing was undertaken on 50 field-collected *An. funestus.* Pyrogram traces showed only the 1014 TTA codon, indicating that they did not have the L1014F (TTA to TTT) or L1014S (TTA to TCA) *kdr* mutations. More detailed analysis is ongoing to ascertain whether any of the resistance phenotype can be mapped to the sodium channel locus.

AChE Gene Sequencing. A 1,290-bp fragment of the AChE gene was sequenced in 10 mosquitoes, 5 bendiocarb resistant and 5 susceptible mosquitoes. The G119S mutation that confers carbamate and organophosphate resistance in *An. gambiae* and *Culex quinquefasciatus* (19) was not detected in these samples, nor was the F455W mutation found in *Cx tritaeniorhynchus* (20). Several indels (from 2 to 45 nucleotides) were observed in the introns, but these were not correlated with resistance or geographical location. A high level of synonymous polymorphism was seen. Sequencing results support the biochemical assays, indicating that there is no target site resistance associated with the observed bendiocarb resistance in *An. funestus* from Chikwawa.

Thus, pyrethroid and bendiocarb resistance in Malawian *An. funestus* is linked to an increase in cytochrome P450s, with the potential for a target site resistance with a mutation distinct from the 1014 residue usually associated with sodium channel target site resistance in *An. gambiae* (7, 8). Similar patterns of pyrethroid resistance occur in *An. funestus* from Mozambique and South Africa (9, 21). It is possible that this resistance front has now extended north from Mozambique, or it may have been selected de novo in Malawi. The resistance patterns in the Malawian and Mozambican populations are similar, with metabolically based resistance to bendiocarb but not organophosphates. Resistance is associated with the up-regulation of a pair of related *CYP6* P450s in both Malawian and Mozambican *An. funestus*. However, there is a major difference in expression levels of the two genes in the two populations. There is a consistently higher level of expression of the *CYP6P9a* gene in Malawi compared with *CYP6P9b*, whereas expression levels of the two genes are comparable in Mozambique. This result suggests that the regulatory mechanism underlying the resistance

Table 3. Summary statistics for polymorphism at the sodium channel gene in susceptible and resistant *An. funestus* from Chikwawa, Malawi

Samples	N	S	Ts	Tv	Singletons	F	h	π (k)	D (Tajima)	D* (Fu and Li)
Susceptible	10	1	0	1	1	90% (*2)	2 (0,2)	0.00022 (0.2)	-1.11^{ns}	-1.24^{ns}
Resistant	10	10	4	6	0	50% (R1)	4 (0,733)	0.0049 (4.48)	1.19^{ns}	1.45*
Total	20	10	4	6	0	55% (*2)	5 (0,653)	0.00441 (4.047)	1.52^{ns}	1.41*

F, frequency of the most common haplotype; h, number of haplotypes (haplotype diversity); N, number of sequences (2n); S, number of polymorphic sites; Ts, transition; Tv, transversion; π, nucleotide diversity (k, mean number of nucleotide differences). Tajima's D and Fu and Li's D statistics: ns, not significant. *$P < 0.05$.

in the two countries may not be identical, although further work is required to confirm this. Up-regulation of members of the *CYP6* rather than *CYPM* family of P450s is consistent with the lack of DDT resistance. Up-regulation of *CYPM2* in West African *An. gambiae* produces high levels of both DDT and pyrethroid resistance (22), and any suggestion that this had been selected in either of the Malawian vectors may have reduced insecticide choice still further.

A comparison of the expression levels of both *CYP6P9a* and *CYP6P9b* in the bendiocarb resistant mosquitoes shows that both genes have significantly lower expression levels in the bendiocarb resistant compared with the permethrin resistant samples. This suggests that these P450s may preferentially metabolize pyrethroids. The involvement of these P450s in cross-resistance between the carbamate and pyrethroid resistances was previously indicated by piperonyl butoxide synergist assays (9).

Vector Control Coverage and Household Surveys. IRS coverage in the five treatment districts was 88.5% in 2010. LLIN use by children aged <5 y was similar in IRS and non-IRS sites (50% and 54%, respectively). Introduction of this level of IRS coverage into a background of moderate to high LLIN use should rapidly produce an obvious reduction in parasite prevalence, as seen operationally in similar high malaria endemic areas such as Bioko, West Africa (23).

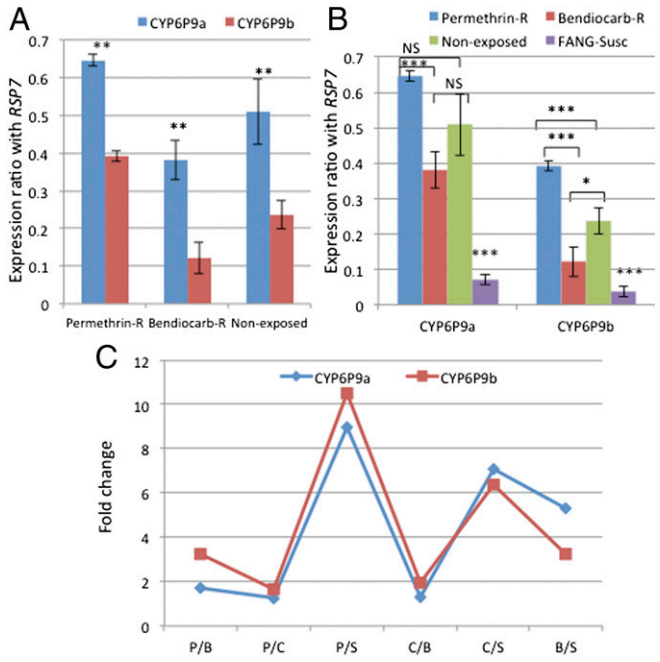

Fig. 4. Transcription profile of candidate genes in the Chikwawa population. (*A*) Comparison of the patterns of gene expression of CYP6P9a and CYP6P9b for each sample. (*B*) Expression ratio of *CYP6P9a* and *CYP6P9b* for sample. The normalized expression ratio of each gene against *RSP7* gene is represented on the vertical axis. (*C*) Fold change of *CYP6P9a* and *CYP6P9b* between different comparisons. P, permethrin resistant; B, bendiocarb resistant; C, nonexposed mosquitoes; S, FANG susceptible strain.

A total of 802 1- to 4-y-old children were surveyed in April 2009 for *Plasmodium falciparum* infection, with a follow up survey in 2010 involving 678 children. The refusal rate was negligible. Combined prevalence of infection across all sites in 2009 was 53.6% [confidence interval (CI) 43.6–63.4%], which was reduced in 2010 to 39.5% (27.6–52.9%). In the LLIN-only sites the reduction in prevalence from 2009 to 2010 was 53.2% (CI 40.4–65.5%) to 40% (23.4–59.1%), whereas the reduction in the IRS plus LLIN sites was from 54.9% (42.3–66.8%) to 38.6% (30.2–47.8%) (Table 4). In 2009 four districts—Kalungama, Kamekete, Makoka, and Mazanduwa—were sprayed by the National Malaria Control Program, with coverage ranging from 60.5% to 86.1%, protecting 126,126 people. IRS was expanded in 2010, and a fifth district (Ambali) was added. IRS coverage in 2010 ranged from 64.5% to 100%. The LLIN use combined for all sites increased from 43.9% to 52.7% [odds ratio (OR) 1.42, CI 0.97–2.09]. The larger increase in LLIN use was seen in the non-IRS districts, increasing from 41.4% to 54.1% (OR 1.69, CI 1.03–2.78) compared with the IRS districts, where LLIN use reduced slightly from 52.2% to 49.7% (OR 0.9, CI 0.63–1.3) (Table 4).

However, for three IRS sites (Kalungama, Kamekete, and Makoka) the CIs indicated that the OR was not inconsistent with 1, suggesting no real change in prevalence despite the increased use of IRS.

Only five sentinel sites (Ambali, Chinkole, Davide, Mazanduw, and Nkhwazi) saw real evidence of a reduction in prevalence. Two of these were IRS districts, with IRS-only starting in Ambali in 2010; the other three sites had only LLINs. Four of the sites had evidence of either *An. gambiae* and/or *An. funestus* becoming resistant to pyrethroids. The resistance status was not assessed in the fifth site. Two of the "real impact" sites (Chinkole with LLINs and Mazanduwa with IRS) had high levels of resistance in *An. funestus*, but *An. gambiae* in Chinkole remained susceptible to deltamethrin.

In three LLIN-only sites (Chapola, Chiwalo, and Nkhoma) there was an increase in prevalence of infection in children aged <5 y (i.e., point estimates of ORs >1 for 2010 relative to 2009). However, none of these estimates are inconsistent with an OR = 1, as evident from the 95% CIs. It would therefore be more accurate to say that there was no evidence of a change in prevalence in these districts.

Within the current dataset the five sites with real reductions in prevalence had as many insecticide resistance reports as the nine sites with no reduction in prevalence, and two of the former had high levels of pyrethroid resistance in *An. funestus*. Hence although pyrethroid resistance in *An. funestus* has gone from undetectable levels in 2007 to moderate or high frequencies throughout the country, we have yet to demonstrate a major epidemiological impact on disease transmission. Pyrethroid susceptibility data, which can be linked to vector control impact indicators, are however needed in larger numbers of sites to be able to address this question with more statistical power.

Sufficient nets were distributed in the initial antenatal clinic campaign in Malawi to theoretically hit the 80% coverage target for pregnant women and children. In practice, net use across all of the sentinel sites within the study area was significantly lower,

Table 4. ITN use, IRS coverage, and prevalence in 1- to <5-y-olds at sentinel sites

	Households sprayed last 12 mo [95% CI](N)		ITN use in children aged <5 y			*P. falciparum* prevalence children aged 1 to 4 y		
Sentinel site	2009	2010	2009	2010	OR	2009	2010	OR (95% CI)
Ambali	0 (58)	92.9 [75.4,98.2] (28)	37.7 [25.4,51.8] (106)	39.1 [26.9,52.8] (64)	1.06 (0.48,2.35)	73.6 (87)	54.8 (42)	0.44 (0.22,0.88)
Chapola	0 (49)	0 (53)	23 [12.8,37.8] (61)	47.4 [33.5,61.8] (78)	3.03 (1.21,7.60)	57.1 (49)	66 (47)	1.45 (0.62,3.40)
Chinkole	0 (70)	0 (50)	76.4 [63.3,85.9] (72)	65.2 [50.5,77.4] (66)	0.58 (0.24,1.38)	61.5 (52)	26.9 (52)	0.23 (0.09,0.57)
Chiwalo	0 (65)	0 (55)	45.8 [33.2,59.1] (72)	45.6 [33.1,58.6] (79)	0.99 (0.47,2.09)	50 (62)	63.2 (57)	1.71 (0.81,3.61)
Davide	0] (45)	0 (48)	38.1 [23.5,55.2] (63)	46.7 [33.5,60.3] (60)	1.42 (0.59,3.45)	27.8 (54)	10.4 (48)	0.3 (0.10,0.90)
Kalungama	64.3 [48.9,77.2] (42)	100 (27)	31.7 [17.9,49.7] (60)	35.8 [22.4,52.0] (53)	1.21 (0.44,3.29)	41.3 (46)	28.3 (46)	0.56 (0.23,1.38)
Kamekete	60.5 [45.3,73.8] (43)	64.5 [46.5,79.2] (31)	66.7 [51.2,79.2] (63)	68.2 [49.8,82.2] (44)	1.07 (0.39,2.92)	52.7 (55)	33.3 (36)	0.45 (0.17,1.18)
Makoka	86.1 [70.6,94.1] (36)	88 [68.6,96.1] (25)	58.3 [40.9,73.9] (48)	50 [31.9,68.1] (68)	(0.25,2.01)	50 (40)	36 (50)	0.56 (0.21,1.48)
Mazanduwa	81.3 [67.7,90.0] (48)	100 (28)	52.5 [37.0,67.5] (80)	58.7 [39.2,75.9] (63)	1.29 (0.47,3.55)	69.2 (65)	41.3 (46)	0.31 (0.13,0.73)
Mgona	0 (49)	0 (52)	15.2 [7.9,27.1] (66)	45.3 [32.3,59.0] (64)	4.64 (1.86,11.61)	22.8 (57)	8.3 (48)	0.31 (0.09,1.03)
Mtanda	0 (36)	0 (37)	49.4 [33.0,65.9] (81)	56.4 [44.0,68.0] (78)	1.33 (0.57,3.09)	66.7 (69)	50 (60)	0.5 (0.23,1.07)
Ndindi	0 (44)	0 (48)	26.3 [16.3,39.5] (76)	68.1 [52.7,80.3] (72)	5.97 (2.46,14.46)	64.7 (68)	57.4 (47)	0.74 (0.34,1.59)
Nkhoma	0 (43)	0 (35)	61.4 [45.5,75.2] (57)	46.9 [31.6,62.8] (64)	0.55 (0.22,1.38)	63.6 (44)	80.6 (36)	2.37 (0.77,7.32)
Nkhwazi	0 (52)	0 (51)	37.7 [25.5,51.7] (69)	63.1 [48.0,76.0] (84)	2.83 (1.22,6.54)	29.6 (54)	11.1 (63)	0.3 (0.11,0.81)
All sites	19.6 [7.2,43.4] (680)	24.8 [10.1,49.3] (568)	43.9 [34.5,53.8] (974)	52.7 [46.9,58.5] (937)	1.42 (0.97,2.09)	53.6 (802)	39.5 (678)	0.57 (0.39,0.83)
Non IRS sites	0 (511)	0 (429)	41.1 [30.2,53.0] (723)	54.1 [47.5,60.6] (645)	1.69 (1.03,2.78)	53.2 (596)	40 (458)	0.59 (0.32,1.06)
IRS sites	72.8 [59.5,83.0] (169)	88.5 [65.7,96.9] (139)	52.2 [38.4,65.6] (251)	49.7 [39.0,60.3] (292)	0.9 (0.63,1.30)	54.9 (206)	38.6 (220)	0.52 (0.32,0.83)

with 40–50% use. Despite this, pyrethroid resistance has appeared and spread rapidly in Malawian malaria vectors. Over the last 3 y the tipping point for resistance selection has been achieved, and the population has shifted dramatically from undetectable levels of resistance by bioassay to >50% of the population carrying one or more resistance genes. This shift in resistance has occurred at scale throughout the country and is not restricted to small areas where selection has been increased, for example by the introduction of pyrethroid-based IRS. Similar shifts have been noted in *Aedes aegypti* in Mexico (24), which is currently underpinning a major outbreak of dengue hemorrhagic fever (25). The speed at which this resistance has appeared and spread emphasizes the importance of high-quality routine screening if resistance is to be detected at an early stage.

The impact of this pyrethroid resistance on our ability to reduce transmission in Malawi still needs to be fully elucidated. Resistance was selected over a period of <3 y and has spread throughout Malawi in *An. funestus*, with a lower but evident

increase in resistance in *An. gambiae*. The continued selection pressure with pyrethoids from the LLINs and further scale up of IRS with pyrethroids in 2010 will maintain and increase the pyrethroid resistance gene frequencies in the field population. If this is combined with favorable rainfall, it is likely to result in a net increase of mosquito population numbers, a large percentage of which will be less susceptible to the vector control in place. The current resistance has not triggered a major increase in parasite prevalence in Malawian children, but it may have reduced the benefit of introducing IRS in several districts. Those districts where IRS was used in Malawi showed similar prevalence levels and trends to the LLIN alone areas, in contrast to Equitorial Guineam, Bioko Island, and Zambezia Porvince, Mozambique, where IRS provided an added benefit in an LLIN background in the initial phases of its introduction (23).

The statistical power required to accurately detect such trends requires large-scale sampling. There are almost no operational control programs that operate annual point prevalence or alternative disease surveillance systems at this kind of scale, alongside appropriate entomological monitoring that could be used to assess trends linked to resistance selection. If these cannot be undertaken operationally, then pragmatically avoiding resistance selection by the judicious use of insecticides where practical in rotations, mosaics, or mixtures will be necessary. The choice of insecticides within these strategies should be guided by the resistance situation on the ground, which should include a clear understanding of the underlying resistance mechanisms, because the cross-resistance patterns may drive the strategy that is required.

Materials and Methods

Area of Study and Mosquito Collection. Fourteen sentinel sites were established in central and southern Malawi (Fig. 1). In 2008 four of these sites (Kamtekete, Kalungama, Makoka, and Mazandua) were in the IRS area. In 2009 a fifth site, Ambali, became an IRS site.

Blood-fed and gravid indoor resting *An. gambiae* and *An. funestus* adult female mosquitoes were routinely collected between 0600–1000 h using an aspirator. Mosquitoes were kept in individual oviposition tubes and induced to lay eggs. Egg batches from females collected at each sentinel site were pooled and reared together to avoid bias from isofemale lines.

Insecticide Bioassays. Insecticide susceptibility assays were carried out using 1- to 3-d-old F_1 adults according to the WHO protocol at 25 ± 2 °C and 70–80% relative humidity (28). Between 5 and 25 adult mosquitoes were exposed to insecticide-treated or control papers (impregnated with insecticide carrier oil) for 1 h and then held with access to 10% sugar solution for 24 h before the percentage mortality was determined. The insecticides and diagnostic dosages tested were bendiocarb (0.1%), deltamethrin (0.05%), lambda cyhalothrin (0.05%), permethrin (0.75%), pirimiphos methyl (0.9%), malathion (5%), and DDT (4%). All papers, with the exception of the pirimiphos methyl papers, which were made immediately before use without carrier oil (27), where purchased from WHO.

Species Identification. All mosquitoes were morphologically identified as belonging to the *An. gambiae* complex or *An. funestus* group (28, 29). Identification to species level was carried out by PCR (30, 31).

Biochemical Assays. Standard biochemical assays for GST, altered AChE, esterase (using both pNPA rate reactions and α- and β-naphthyl acetate end point assays), and monooxygenase (P450) quantification were carried out according to the methods described previously (32) using 30 1- to 3-d-old female *An. funestus* adults from the Chikwawa mixed F_1 mosquito pool. The *An. gambiae* Kisumu strain (33) was used as the susceptible control sample, because susceptible *An. funestus* were not available. A two-sample *t* test was used to compare results between strains after adjustments for total protein content. The adjusted mean enzyme level/activity of Chikwawa were compared with the corresponding Kisumu means.

Sodium Channel Gene Sequencing for Detection of *kdr* Mutations. The target site-based pyrethroid\DDT resistance known as *kdr* was assessed by sequencing a fragment of the sodium channel gene in *An. funestus*. This spanned the 1014 codon in exon 20 (domain II, segment 6) associated with *kdr* resistance in *An. gambiae* (7, 8). It was assessed in pyrethroid resistant and susceptible mosquitoes from Chikwawa to correlate the presence of any mutation with pyrethroid resistance. DNA was extracted using the LIVAK method (36) and amplified using primers Kdrfun-F GTT CAA TGA AGC CCC TCA AA and Kdrfun-R CCG AAA TTT GAC AAA AGC AAA. The PCR was carried out using 10 pmol of each primer and 30 ng of genomic DNA as template in 25-μL reactions containing 1× Kapa Taq buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U Kapa Taq (Kapa Biosystems). The cycle parameters were 1 cycle at 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 57 °C for 30s, and elongation at 72 °C for 1 min; followed by 1 cycle at 72 °C for 10 min. Sequences were aligned using ClustalW (37), whereas haplotype reconstruction and polymorphism analysis was done using DnaSP v5.0 (36). The phylogenetic neighbor-joining trees were constructed using MEGA 4.0 (37).

AChE Sequencing. Target site-based organophosphate and\or carbamate resistance was assessed by sequencing a fragment of the AChE gene (accession no. DQ534435). This spanned the G119S and F455W mutations previously associated with carbamate resistance (19, 38). The region was amplified and sequenced in individual carbamate resistant and susceptible mosquitoes from Chikwawa. The following primers were used for the PCR amplification: *AChE* forward CCA CTG TCG GAG GAC TGT CT and *AChE* reverse CGT TAA CGT ACG GGT CGA GT. The PCR was carried out using 10 pmol of each primer and 30 ng of genomic DNA (gDNA) as template in 25-μL reactions containing 1× Kapa Taq buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U Kapa Taq. The cycle parameters were as follows: 1 cycle at 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and elongation at 72 °C for 1 min; followed by 1 cycle at 72 °C for 10 min.

Genotyping *An. funestus* Samples for Detection of 1014 *kdr* and 119 *Ace-1* Mutations Using Pyrosequencing. Pyrosequencing was used to genotype the positions of the known 1014 *kdr* and 119 *Ace-1* mutations as described for kdr genotyping in *Culex quinquefasciatus* (39). The pyrosequencing AB software was used to design three sequence-specific primers for *Ace-1* and *kdr* mutations (the L1014F and L1014S *kdr*-variants are detected in the same assay). The sequences for genotyping analysis and the dispensation order for both reactions are the same as previously described (40). Target DNA fragments for *kdr* and *Ace-1* were first amplified by PCR using the forward and biotinylated reverse primers. The PCR contained forward and biotinylated reverse primers (10 pmol), 1× HotStarTaq buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 U HotStarTaq (Qiagen), and 10 ng gDNA. The parameters for amplification were as follows: 1 cycle at 95 °C for 5 min; 50 cycles of 94 °C for 20 s, 57 °C for 30 s, and elongation at 72 °C for 20 s; followed by 1 cycle at 72 °C for 5 min.

Pyrosequencing reactions were performed as previously described (40) according to the manufacturer's instructions using the PSQ 96 SNP Reagent Kit (Biotage) and the sequencing primer. The genotype was automatically determined by the pyrosequencer.

Transcription Profiling of Relevant P450 Genes. Quantitative PCR analysis of mosquitoes for two duplicated P450 genes (CYP6P9a and CYP6P9b) previously found to be associated with pyrethroid resistance in the FUMOZ-R strain of *An. funestus* (17) and in field samples of *An. funestus* from Mozambique (21) were carried out to assess whether they were also over expressed in the Malawian *An. funestus* field samples. The GeXP genetic analysis system from Beckman Coulter was used according to the protocol in ref. 17. RNA was extracted using the Picopure RNA isolation kit (Arkturis) from three batches of 10 females from Chikwawa, respectively, for resistant to permethrin, bendiocarb resistant, and for females not exposed to insecticides (as control for the population). The same was done for the laboratory susceptible FANG strain of *An. funestus* (originally from Angola) (41). The FANG mRNA was stored in RNA later, hence whole insect samples were not available for the biochemical assays. RNA quality was assessed using the Agilent 2100 Bioanalyzer. The following primers were used: CYP6P9a\b forward AGG TGA CAC TAT AGA ATA CAA TGT GAT AAA CGA AAC ACT TCG CAA (common to both CYP6p9 copies and spanning the intron); CYP6P9a reverse GTA CGA CTC ACT ATA GGG ACT TTA TTA TAG ATT GGA AGT ATC TCA (expected product of 490 bp); CYP6P9b reverse GTA CGA CTC ACT ATA GGG ACT ACA AAA ACC CCT TCC GCT GCA CC (expected product of 504 bp). The expression level of the *RSP7* ribosomal gene, shown to be consistent and with no differential expression between susceptible and resistant (17, 18), was used to normalize for variation in total cDNA concentration between samples. A two-sample *t* test was used to compare the results between samples.

Household Surveys. Cross-sectional household surveys were carried out at each sentinel site on a random sample of 140 1- to 4-y-old children. Households were selected from strata formed by dividing sentinel sites into quadrants from which participants were selected, to ensure the greatest

geographical spread within the site. Diagnostic tests with ICT malaria rapid tests (ICT, Global Diagnostics) were used to assess the *P. falciparum* infection status of each of the sampled children. Participants who tested positive were offered treatment with Coartem (Novartis) (artemether and lumefantrine) according to Malawi's national malaria treatment guidelines.

Household surveys were carried out in April at the end of the 2009 and 2010 malaria transmission seasons. The sentinel site-specific sample size was calculated to provide evidence at the 5% significance level of an absolute reduction in *P. falciparum* prevalence of 20% following the intervention. Prevalence and 95% CIs for each sentinel site were estimated, taking account of clustering by sentinel site using the statistical software package STATA, release 10 (StataCorp).

Sentinel sites were considered the primary sampling unit. Logistic regression, allowing for complex survey designs, was performed to estimate the mean effect of the intervention on prevalence compared with baseline prevalence of infection in different years.

Ethics. Ethics approval for this work was obtained from the ethics committees of the College of Medicine (COMREC) in Malawi and Liverpool School of Tropical Medicine in the United Kingdom.

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