Rapid selection of a pyrethroid metabolic enzyme CYP9K1 by operational malaria control activities

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Since 2004, indoor residual spraying (IRS) and long-lasting insecticide-impregnated bednets (LLINs) have reduced the malaria parasite prevalence in children on Bioko Island, Equatorial Guinea, from 45% to 12%. After target site-based (knockdown resistance; *kdr*) pyrethroid resistance was detected in 2004 in *Anopheles coluzzii* (formerly known as the M form of the *Anopheles gambiae* complex), the carbamate bendiocarb was introduced. Subsequent analysis showed that *kdr* alone was not operationally significant, so pyrethroid-based IRS was successfully reintroduced in 2012. In 2007 and 2014–2015, mass distribution of new pyrethroid LLINs was undertaken to increase the net coverage levels. The combined selection pressure of IRS and LLINs resulted in an increase in the frequency of pyrethroid resistance in 2015. In addition to a significant increase in *kdr* frequency, an additional metabolic pyrethroid resistance mechanism had been selected. Increased metabolism of the pyrethroid deltamethrin was linked with up-regulation of the cytochrome P450 CYP9K1. The increase in resistance prompted a reversion to bendiocarb IRS in 2016 to avoid a resurgence of malaria, in line with the national Malaria Control Program plan.

cytochrome P450 | resistance | pyrethroid | malaria | control

Malaria prevalence in Africa has been halved since 2000, with 80% of the reduction being the result of the use of vector control (1). This reduction is primarily attributable to pyrethroid-treated bed nets and indoor residual spraying (IRS). Pyrethroid resistance in mosquito populations is at a critical tipping point in Africa, threatening the progress made in reducing malaria transmission (2, 3).

The most prevalent pyrethroid resistance mechanisms in *Anopheles* affect the voltage-gated sodium channel target site of the insecticide or its metabolic degradation pathway. Additional resistance mechanisms include epicuticular changes, which slow the uptake of pyrethroids (4). Knockdown resistance (kdr) mutations at the target site typically alter the insecticide binding affinity, and the most common in *Anopheles* are mutations L1014F and L1014S in the voltage-gated sodium channel (5, 6). In addition, the mutation N1575Y combined with L1014F strengthens resistance to pyrethroids and dichlorodiphenyltrichloroethane (DDT) (7, 8), which used to be one of the most widely used insecticides for malaria control and is still being used in several parts of the world. Kdr resistance alone has not been correlated with operational control failure in *Anopheles gambiae*. The accumulation of kdr and metabolic resistance mechanisms, however, have resulted in much higher pyrethroid resistance levels, which may have an operational impact (9–11).

Overexpression of detoxification enzymes such as cytochrome P450 monooxygenases (henceforth "P450s") is frequently found in association with metabolically based insecticide resistance in *An. gambiae* (12). The P450 genes *Cyp6M2* and *Cyp6P3* are often overexpressed in resistant *An. gambiae* (13), and both metabolize permethrin and deltamethrin (14, 15). *Cyp6Z2* is also sometimes overexpressed, but binds/sequesters rather than metabolizes the pyrethroids permethrin and cypermethrin (16).

Analysis of metabolic-resistance mechanisms typically involves transcriptomic comparisons between resistant laboratory strains or field-caught populations and laboratory susceptible strains (17), with occasional examples of spatial or temporal comparisons made between field mosquitoes that differ in resistance phenotype (18). In Burkina Faso, significant changes in expression of P450s were linked to an increase in pyrethroid resistance (10), and, in Benin, expression of *Cyp6M2* and *Cyp6P3* increased in *An. gambiae* after mass distribution of long-lasting insecticide-treated nets (LLINs) (19). *Kdr* frequencies were unchanged in both cases. An association between pyrethroid use and an increase in the relative frequency of *kdr* 1014F and a nearly fixed X-linked haplotype containing *Cyp9K1* was demonstrated in a genome-wide study of mosquitoes from Mali (20), but the role of this P450 gene in resistance has not been established.

Before the Bioko Island Malaria Control Project (BIMCP) and the Ministry of Health and Social Welfare established the malaria control program in 2004, Bioko had one of the highest entomological inoculation rates recorded in Africa, at ∼1,200 infectious bites per person per year (21). In 2004, there were three major mosquito vector species on the island: *Anopheles*

Significance

Eliminating malaria from islands should, in theory, be easier than eliminating malaria from countries in mainland Africa because of restricted movement of insects and people between treated and untreated areas. The example of Bioko Island, where the entomological inoculation rate in 2004 was among the highest in Africa, demonstrates how difficult this can be. Vector control has eliminated two of the four vector species, and malaria has been dramatically reduced. This study demonstrates rapid evolution of resistance following reintroduction of pyrethroid-based control interventions, with the selection of a P450-based mechanism (CYP9K1). Urban malaria, movement of infected people from the mainland, and selection of this pyrethroid-resistance mechanism in addition to knockdown resistance has impeded progress and forced a change to nonpyrethroid indoor residual spraying.

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funestus and the M and S forms of *An. gambiae*, now elevated to species as *An. gambiae* (S) and *Anopheles coluzzii* (M) (22). Malaria prevention efforts used pyrethroid-impregnated LLINs and IRS, with the carbamate bendiocarb or the pyrethroid deltamethrin (see Fig. 1 for timeline). A country-level operational implementation of the World Health Organization (WHO)-recommended Global Plan for Insecticide Resistance Management (GPIRM) was established in 2012 (23), with insecticide resistance monitoring providing robust data to support evidence-based insecticide resistance management strategies.

Pyrethroid-based IRS was used when the program was initiated, but resistance was quickly detected. Bendiocarb-based IRS was instigated in the second spray round, but this switch increased cost and reduced the effective longevity of the IRS. A comprehensive study of the resistance mechanisms present in *An. gambiae* from Bioko was conducted in 2011; *kdr* 1014F genotypes were at high frequencies (28.5% FF, 57.14% LF), with no significant metabolic resistance (23). With *kdr* alone expected to have no operational impact on pyrethroid-based IRS efficacy, the BIMCP reintroduced pyrethroids in 2012.

In 2015, bioassays indicated that pyrethroid resistance frequency and level were increasing rapidly, which triggered detailed analysis of the local vector population composition and insecticide-resistance mechanisms to determine the appropriate vector control interventions for 2016.

Results

Bioassays and Genotypes. A timeline for introduction of different vector control activities in Bioko, overlaid on the annual malaria prevalence figures for children 2–14 y old, is given in Fig. 1. There were some issues with the pyrethroid IRS formulation in 2013, which were corrected in 2014, but there were no indications from bioassay data that resistance increased from 2013 to 2014.

The levels of insecticide resistance increased in 2015 compared with earlier years, with mosquitoes from two field sites (hospital and industrial) having <30% mortality after exposure to the WHO discriminating dosage of deltamethrin but remaining susceptible to bendiocarb (mortality rate > 99%; Table S1).

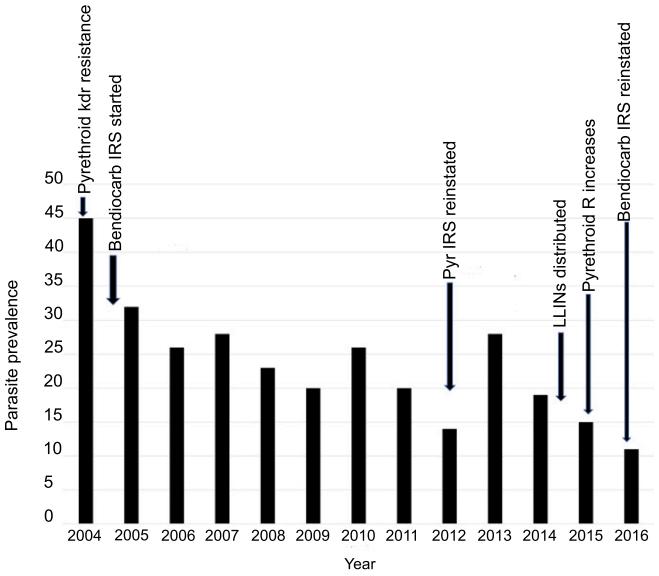

Fig. 1. Timelines for vector-control events and *Plasmodium falciparum* parasite prevalence data in 2–14-y-old children in 2004–2016. Arrows indicate major vector control events over time in Bioko. Pyr, pyrethroids; R, resistance.

From the two sites where resistance was analyzed in detail, all mosquitoes were identified as *An. coluzzii* (Table 1). All samples collected in 2015 were homozygous for the L1014F *kdr* mutation, but the L1014S and N1575Y and AChE G119S mutations were not detected. This finding represents a significant increase in *kdr* from 2011 (28.5% 1014F/F; $\chi^2_2 = 46.5$, $P = 8 \times 10^{-11}$; Table 1). Annual island-wide surveys of *kdr* confirmed the increase in frequency (Fig. 2) from 2011 to 2015.

Gene Expression. Several genes were overexpressed when the Bioko field-collected populations were compared with the insecticide-susceptible laboratory colonies. Of the 48 genes that were significantly overexpressed in all comparisons performed, four were P450s: *Cyp6P4*, *Cyp9K1*, *Cyp6Z1*, and *Cyp6Z2* (Fig. 3 and Dataset S1). The up-regulation of each gene was assessed by quantitative RT-PCR (qPCR). We also included *Cyp6P3* in the analysis as a strong pyrethroid metabolizer that narrowly missed statistical significance in one of the four comparisons, in addition to the other known strong metabolizer, *Cyp6M2*, even though this was not consistently differentially expressed. The qPCR results confirmed the statistically significant up-regulation of these P450 genes in most comparisons, with the exception of *Cyp6M2* (Fig. 4*A*).

We then compared expression levels of the same P450 genes between the populations collected in 2011 and 2015 (Fig. 4*B*). There was a significant and substantial elevation of expression of *Cyp9K1* and, to a lesser extent, of *Cyp6P3* in 2015 samples compared with 2011. *Cyp6P4* showed a significant but slight decrease in one site. The expression of the other P450s was unchanged.

Functional Expression and Characterization of CYP9K1. As the role of the *Cyp9K1* gene in insecticide resistance had not been evaluated previously to our awareness, CYP9K1 and *An. gambiae* cytochrome P450 reductase (CPR) were functionally coexpressed in *Escherichia coli* and directed to the inner bacterial membrane by using the leading sequences ompA and pelB, respectively. The reduced carbon monoxide-difference spectrum obtained indicated that CYP9K1 was expressed predominately in its active P450 form, characteristic of a well-folded and functional enzyme (Fig. S1*A*). The isolated bacterial membranes contained 1.5 μM P450, whereas the measured P450 reductase activity (AgCPR) was 136.5 μmol cytochrome *c* reduced/min/g protein. CYP9K1 showed high activity for the fluorescent substrate 7-ethoxycoumarin (Table 2).

CYP9K1 was able to metabolize 32% of the deltamethrin applied following a 2-h incubation (Table 2). Deltamethrin metabolism followed Michaelis–Menten kinetics (K_m = 28.2 μM and V_{max} = 0.57 pmol deltamethrin/min/pmol P450; Fig. S1*B*). CYP9K1 likewise metabolized pyriproxyfen, as observed by the NADPH-dependent depletion of this insecticide (Table 2) and the formation of two metabolites, which eluted at 6.2 min and 7.4 min, respectively (24). In contrast, bendiocarb was not metabolized by CYP9K1, with no substrate depletion after a 20-min incubation, after which spontaneous, non–P450-specific, degradation occurred.

Discussion

Bioko Island is implementing a country-level operational implementation of the GPIRM with evidence-based malaria vector control. A robust program for insecticide resistance monitoring has been in place since 2004 (23). In 2011, pyrethroids were still operationally functional, and the BIMCP rotated back to pyrethroid IRS in 2012 after 7 y of bendiocarb use to maintain levels of disease control, reduce cost, and reduce the selection pressure on bendiocarb. Three years later, after bioassays signaled a change in pyrethroid-resistance status of the local vector (deltamethrin mortality rate of 25.7% in 2015 vs. 40% in 2011) but detected no difference in the susceptibility to bendiocarb (Table S1), a detailed insecticide-resistance analysis was undertaken. The frequency of

Table 1. Molecular form composition and frequencies of target-site resistance mutations in field-caught *An. gambiae s.l.* populations collected in Bioko

Population	Genotypes			Target-site allele frequency, %*			
	No. of specimens	Molecular form, %		No. of specimens	1014F/F	1014F/L	10114L/L
		M/M	S/S				
Malabo 2004†	—	36	64	4,500	14	41	45
Malabo 2011	75	100	0	75	28.5	57.14	14.3
Malabo hospital 2015	16	100	0	16	100	0	0
Malabo industrial 2015	16	100	0	16	100	0	0

M, *An. coluzzii* (M form); S, *An. gambiae s.s.* (S form).
*Data from Hemingway et al., 2013 (23).
†Mutations L1014S and N1575Y on the voltage-gated sodium channel, as well as mutation G119S on acetylcholinesterase, were absent in all alleles ($n > 200$) genotyped in both 2011 and 2015. F/F, individuals homozygous for mutation L1014F on the voltage-gated sodium channel; F/L, heterozygotes; L/L, homozygous individuals for the WT (i.e., susceptible) allele.

the target site pyrethroid resistance mutation L1014F was dramatically higher in 2015 (100% in 2015 and 57% in 2011 at the same collection sites), but the *Anopheles* pyrethroid *kdr*-based resistance enhancing mutation N1575Y and the AChE resistance mutation (G119S) were not found. The P450s *Cyp6P4*, *Cyp9K1*, *Cyp6Z1*, *Cyp6Z2*, and *Cyp6P3* were overexpressed in *An. coluzzii* from Bioko compared with the susceptible colonies (N'gusso and Kisumu), indicating a possible link to the increased pyrethroid resistance. Specific P450 genes are repeatedly implicated in pyrethroid resistance in *An. gambiae* and *An. coluzzii* populations (13), and, in some cases, single P450s have been demonstrated to confer resistance via heterologous expression in *Drosophila* (9). In resistant mosquito field populations, however, multiple P450s are typically overexpressed to moderate levels, in contrast with *Drosophila* and agricultural pest examples, in which resistance is often attributed to dramatic elevation of single P450s (25, 26).

In the "hospital 2015" and "industrial 2015" *An. coluzzii* populations, there was a 6–10-fold increase in expression of *Cyp9K1*, and lower, but significant, overexpression of *Cyp6P3* compared with mosquitoes sampled from the same regions in 2011. Selection by pyrethroid-based IRS and more recently LLINs in Bioko may have given rise to this metabolic resistance coupled with an increase in *kdr* frequency. Resistance is likely to have arisen de novo in *An. coluzzii* on the island, as there is no evidence over several years of *An. funestus* or *An. gambiae* s.s. reinvading from the mainland after local elimination, coupled with previous evidence of a de novo local origin of *kdr* 1014F (27). The situation on Bioko is one of very few examples of documented changes in metabolic gene expression linked to control interventions.

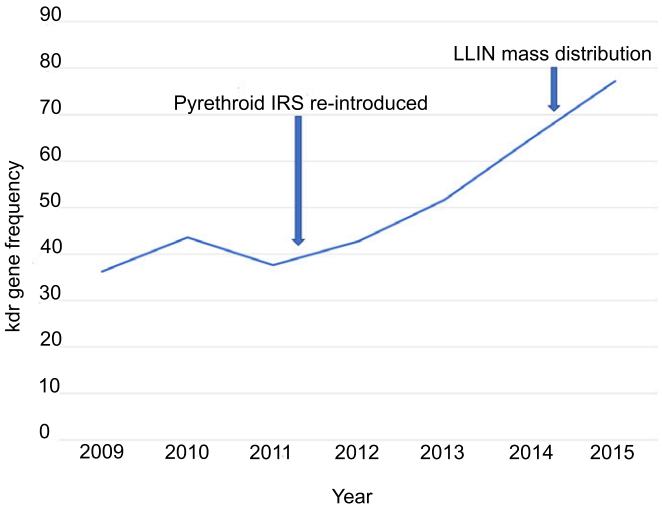

Fig. 2. Frequency of the L1014F kdr mutation in *A. coluzzii* on Bioko Island, 2009–2015. Data generated from combined routine island-wide annual entomological surveillance data and the more detailed studies undertaken in 2011 and 2014 at two sites in Malabo. Arrows indicate time points at which new pyrethroid-based operational IRS and LLIN control interventions occurred.

Relatively few candidate genes in *An. gambiae* spp. have been subjected to functional validation, without which a definitive causal link with insecticide resistance cannot be made. CYP9K1 was able to metabolize deltamethrin (K_{cat}, 0.57 min^{-1}; K_m, 28.2 μM), albeit with lower in vitro catalytic efficiency compared with other major P450s linked with resistance, CYP6P3 (K_{cat}, 1.8 min^{-1}; K_m, 5.9 μM) (14) and CYP6M2 (K_{cat}, 1.1 min^{-1}; K_m, 2 μM) (15). However, the actual in vivo contribution of each P450 to the resistance phenotype is a more complex issue, as factors such as tissue localization of each enzyme may affect the resistance phenotype. Moreover, combined metabolic activity of more than one P450 on pyrethroid substrates and their metabolites has been reported (28, 29), which might also be the case here, perhaps mediated by synergistic interaction with the other overexpressed enzyme, CYP6P3. An interesting consideration for alternative interventions is that CYP9K1 was also able to metabolize the juvenile hormone analog pyriproxyfen, in line with other major cytochrome P450 metabolic enzymes (30). This result is a concern because pyriproxyfen is being combined with pyrethroids in some next-generation LLINs. CYP9K1 did not metabolize bendiocarb and CYP6P3 has very low catalytic efficiency for bendiocarb (9), consistent with the absence of phenotypic resistance.

It has been hypothesized that the coevolution of P450s and target-site resistance mutations can lead to high levels of resistance that challenge operational efficacy of pyrethroids (20, 31). When metabolic pyrethroid resistance was first selected in Africa, *Cyp9K1* did not appear to be a strong indicator of metabolic resistance in *An. gambiae*, a pattern that may now be changing. In addition to the present data from Bioko, *Cyp9K1* was found to be up-regulated in DDT-resistant *An. gambiae* and *An. coluzzii* populations from Cameroon possessing *kdr* 1014F (32) and also in deltamethrin-resistant but *kdr*-free *Anopheles parensis* from Uganda (33). A section of the X-chromosome that included *Cyp9K1* was detected as being under positive selection, together with the L1014F *kdr* mutation, in *An. coluzzii* populations from Mali after an increase in insecticide pressure by the

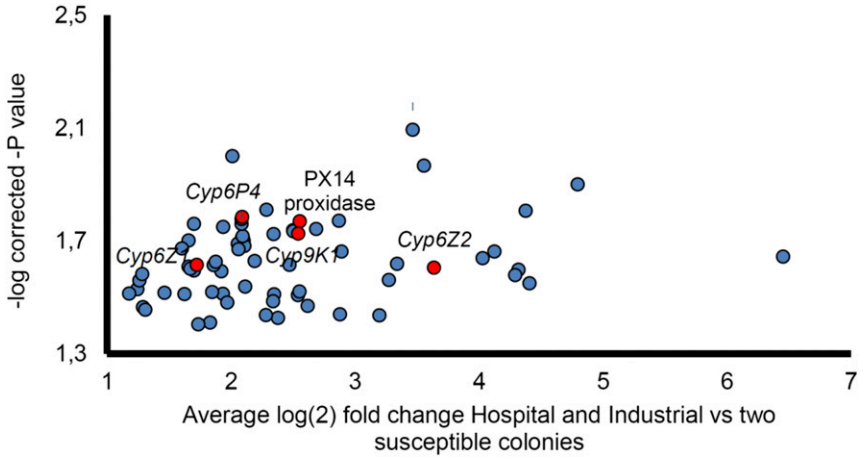

Fig. 3. Significantly up-regulated transcripts found in field-caught resistant populations. Transcripts were found to be significantly up-regulated in the hospital 2015 and industrial 2015 populations in all comparisons performed with the two susceptible laboratory colonies N'gusso and Kisumu. The average log(2) FC between resistance and susceptible replicates is shown. Detoxification genes are highlighted in red.

widespread use of insecticide-treated nets with pyrethroids (20). This growing body of evidence suggesting a role for CYP9K1 in resistance is now further supported by demonstration of functional expression and metabolic activity.

In 2004, the majority of malaria mosquito vectors collected on Bioko were *An. gambiae s.s.* In 2015, *An. coluzzii* and *Anopheles melas* were the only vector species collected. Hence, the vector-control activities may now have eliminated *An. funestus* and *An. gambiae s.s.* from the island. In Mali, *Cyp9k1* was selected as an *An. coluzzii* haplotype from standing variation, rather than via introgression from *An. gambiae* (20). On Bioko, the simultaneous detection of increased *Cyp9k1* expression, coupled with an increase in *kdr* 1014F frequency and higher levels of pyrethroid resistance, suggests that the two mechanisms may act synergistically.

Conclusion

The change in the resistance profile of *An. coluzzii* observed over a period of 3 y after pyrethroid IRS was reinstated is mediated by target site and metabolic resistance mechanisms involving a newly validated cytochrome P450 pyrethroid metabolizer, CYP9K1. In line with the national evidence-based resistance management plan, a recommendation that a nonpyrethroid IRS formulation should be used in 2016 was made. The susceptibility of *An. coluzzii* and *An. melas* vectors to organophosphates and carbamates indicates that bendiocarb or the longer-lasting formulation of Actellic 300CS (34) are the best alternatives.

Materials and Methods

Malaria Prevalence Surveys. Annual malaria prevalence surveys in children 2–14 y of age were undertaken as described by Bradley et al. (35). Informed consent was obtained from all participants. Annual parasite prevalence surveys are covered by ongoing IRB clearance through the Ministry of Health and Marathon Oil Malaria elimination programme in Equatorial Guinea, which has been in place since 2003.

Mosquito Collections and Bioassays. Island-wide routine entomological monitoring was undertaken annually from 2009 to 2015 to assess species composition and the frequency of the kdr-pyrethroid resistance mechanism.

More intensive analysis of resistance was undertaken in 2004, 2011, and 2014 at more limited sites in response to changes in resistance levels detected in routine surveys. For the intensive surveys, immature *Anopheles* were collected from the same sites each year: the grounds of the general hospital in the Ela Nguema district of Malabo (03°45.583 N, 008°47.364 E; referred to as "hospital") and a former industrial site along the harbor (03°45.325 N, 008°46.003 E; "industrial"). Both sites are in the center of Malabo, close to housing. Field-collected larvae were reared to adulthood, and 3-d-old non–blood-fed females were bioassayed by using the WHO susceptibility tests (36). Briefly, mosquitoes were exposed to insecticide-impregnated papers (0.05% deltamethrin or 0.1% bendiocarb; WHO recommended doses) for 1 h, and mortality rates were recorded 24 h later.

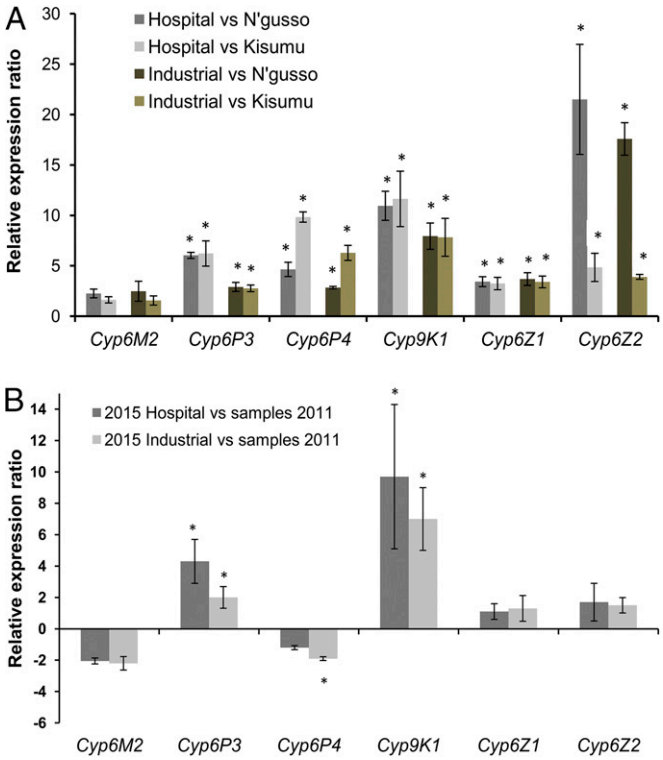

Fig. 4. Expression analysis of selected P450s by qPCR. (*A*) qPCR comparisons of selected P450 transcripts between hospital 2015 and industrial 2015 *An. gambiae* field populations from Bioko vs. the two susceptible laboratory colonies (N'gusso, Kisumu). Error bars represent the SE based on four biological replicates. Asterisk indicates a statistically significant difference in expression levels between the tested samples (Mann–Whitney *U* test, $P < 0.05$). (*B*) Comparison of P450 transcript expression levels between field-caught populations in 2015 (hospital and industrial) vs. samples collected in 2011. Error bars represent the SE based on four biological replicates. Asterisk indicates a statistically significant difference in expression levels between the tested samples (Mann–Whitney *U* test, $P < 0.05$).

Table 2. Activity of recombinant CYP9K1 against model substrates and insecticides

Substrate	Specific activity/depletion of parental compound, %	
	−NADPH	+NADPH
7-Ethocycoumarin	No activity	10.6 ± 1.1
Deltamethrin	No depletion	32.0 ± 3.24
Bendiocarb	No depletion	No depletion
Pyriproxyfen	No depletion	38.0 ± 1.3

Activities are given in picomoles of 7-hydroxycoumarin per minute per picomole of P450 for the substrate 7-ethocycoumarin and as percentage depletion of the parental insecticides under assay conditions.

Molecular Genotyping for Species Identification and Target Site Resistance Mutations. *An. gambiae s.l.* were identified by using a PCR-based diagnostic assay (37) with *An. gambiae s.s.* and *An. coluzzii*, two members of this complex subsequently separated by using the short interspersed element (SINE) PCR method (38). SINE PCR is needed because the two species are morphologically indistinguishable. TaqMan assays with probes detecting the mutant resistant allele and the WT susceptible allele were used to screen for the presence of three *kdr* mutations (L1014F, L1014S, N1575Y) and the G119S AChE mutation conferring resistance to pyrethroids and DDT and to organophosphates, respectively (7, 39).

Analysis of Gene Expression (Metabolic Resistance). Three-day-old field-caught, non–blood-fed female mosquitoes were compared with adult females of the same age from the N'gusso (*An. coluzzii*) and Kisumu (*An. gambiae s.s.*) insecticide-susceptible laboratory colonies. Total RNA was extracted from four replicate batches of 10 mosquitoes from each group (N'gusso, Kisumu, hospital, and industrial) by using a PicoPure RNA isolation kit (Thermo Fisher Scientific) and treated with DNase using the Qiagen RNase-free DNase kit according to the manufacturer's instructions. RNA quantity and quality were evaluated with a NanoDrop spectrophotometer (Thermo Fisher Scientific) and a model 2100 Bioanalyzer (Agilent Technologies). RNA pools from each group were amplified and labeled by using the Low Input Quick Amp Labeling Kit (Agilent Technologies). The Agilent Agam15k array (40) was used for dual-color hybridizations (hospital vs. N'gusso, hospital vs. Kisumu, industrial vs. N'gusso, and industrial vs. Kisumu). Each mosquito population comparison was repeated four times.

Agilent Feature Extraction Software v12 and GeneSpring v13 were used for the analysis of the microarray data. Significance was determined by a one-sample *t* test (against the null hypothesis, which was a ratio of field/colony sample expression of 1) with the *P* value threshold set at $P < 0.05$ following correction for multiple testing (Benjamini–Hochberg method). A fold change (FC) criterion of FC > 2 or FC < −2 (for underexpressed probes) was used.

RNA was reverse-transcribed by using SuperScript III (Invitrogen) and oligo(dT)20 primers to produce cDNA. Expression of candidate genes from the microarray analysis was validated by qPCR (primers listed in Table S2) and normalized using the Ribosomal S7 (AGAP010592) and Elongation Factor (AGAP005128) reference genes (10). Mosquito samples from 2011, before the reintroduction of deltamethrin in 2012, were included in this analysis to allow direct comparisons between temporally separated field-caught samples. Experiments were performed by using four biological replicates from each treatment group and two technical replicates for each reaction. Relative expression analysis was performed according to ref. 41.

Functional Expression of CYP9K1. cDNA from the adult hospital population was used as a template to amplify the full-length *Cyp9K1* by using a proof reading polymerase (Kappa Taq DNA polymerase, long range) and primers listed in Table S2. The hospital strain was used because it had the highest levels of CYP activity. Conditions of amplification were 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 25 s, annealing at 54 °C for 15 s, and extension at 72 °C for 2 min, followed by a final extension step for 5 min. Primers used for the amplification introduced a EcoRI restriction site before the start ATG codon and an XbaI site after the stop codon. The purified PCR product (NucleoSpin PCR and gel extraction purification; Macherey–Nagel) was ligated into the pCWompA2 expression plasmid (16) and sequenced. For protein expression, the strategy described in ref. 42 was followed by using *E. coli* BL21STAR cells cotransformed with the pCWompA2-*Cyp9K1* P450 and the pACYC-AgCPR (expression plasmid for the *An. gambiae* CPR). Membrane preparations were tested for total protein concentration (43), P450 concentration (44), and CPR activity (45).

Cytochrome P450 Activity and Insecticide Metabolism Measurements. Activity of recombinant CYP9K1 against the fluorogenic P450 substrate 7-ethoxycoumarin and eight different luciferin-conjugated substrates (P450 Glo proluciferin substrates Luciferin-H, Luciferin-ME, Luciferin-CEE, Luciferin-H EGE, Luciferin-PFBE, Luciferin-PPXE, Luciferin-ME EGE, and Luciferin-IPA; Promega) was measured (42).

Metabolism of deltamethrin, bendiocarb, and the insect growth regulator pyriproxyfen by CYP9K1 was measured. Deltamethrin (10 μM Fluka; Pestanal), bendiocarb (10 μM Fluka; Pestanal), and pyriproxyfen (50 μM Fluka; Pestanal) were incubated with 25 pmol recombinant CYP9K1 and 200 pmol b5 (AgCytb5; GB AY183376) in 100 μL Tris·HCl buffer (0.2 M, pH 7.4), containing 0.25 mM $MgCl_2$. The incubation was performed in the presence and absence of an NADPH generating system: 1 mM glucose-6-phosphate (Sigma-Aldrich), 0.1 mM NADP+ (Sigma-Aldrich), and 1 U/mL glucose-6-phosphate dehydrogenase (G6PDH; Sigma-Aldrich). Reactions were incubated at 30 °C, agitated at 9,600 × *g* oscillation, and stopped at different time points by using 100 μL acetonitrile. The quenched reactions were centrifuged at 9,600 × *g* (MicroCL 17R centrifuge; Thermo Scientific) for 10 min, and 100 μL of the supernatant was assessed by HPLC by using a UniverSil HS C18 (250 mm 5 μm) reverse-phase analytical column (Fortis). Reactions with deltamethrin were run with an isocratic mobile phase of 10% H_2O and 90% acetonitrile with a flow rate of 1 mL/min for 20 min. Deltamethrin elution was monitored at 225-nm absorbance at a retention time of 9.8 min. Reactions with bendiocarb were run with an isocratic mobile phase of 65% H_2O and 35% acetonitrile with a flow rate of 1 mL/min for 20 min. Bendiocarb elution was monitored at 205-nm absorbance at a retention time of 17.2 min. Reactions with pyriproxyfen were run with an isocratic mobile phase of 30% H_2O and 70% acetonitrile with a flow rate of 1.5 mL/min for 25 min. Pyriproxyfen elution was monitored at 232-nm absorbance and at a retention time of 18.1 min. For kinetics of deltamethrin, varying concentrations of substrate were used (0.5–150 μM). Rates of deltamethrin turnover were plotted vs. deltamethrin substrate concentrations, and the kinetic parameters K_m and V_{max} were determined by using SigmaPlot 12.0 (Systat Software) by fitting to the Michaelis–Menten equation.

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