Identification and validation of a gene causing cross-resistance between insecticide classes in *Anopheles gambiae* from Ghana

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In the last decade there have been marked reductions in malaria incidence in sub-Saharan Africa. Sustaining these reductions will rely upon insecticides to control the mosquito malaria vectors. We report that in the primary African malaria vector, *Anopheles gambiae sensu stricto*, a single enzyme, CYP6M2, confers resistance to two classes of insecticide. This is unique evidence in a disease vector of cross-resistance associated with a single metabolic gene that simultaneously reduces the efficacy of two of the four classes of insecticide routinely used for malaria control. The gene-expression profile of a highly DDT-resistant population of *A. gambiae s.s.* from Ghana was characterized using a unique whole-genome microarray. A number of genes were significantly overexpressed compared with two susceptible West African colonies, including genes from metabolic families previously linked to insecticide resistance. One of the most significantly overexpressed probe groups (false-discovery rate-adjusted $P < 0.0001$) belonged to the cytochrome P450 gene *CYP6M2*. This gene is associated with pyrethroid resistance in wild *A. gambiae s.s.* populations) and can metabolize both type I and type II pyrethroids in recombinant protein assays. Using in vitro assays we show that recombinant CYP6M2 is also capable of metabolizing the organochlorine insecticide DDT in the presence of solubilizing factor sodium cholate.

Recent successes in reducing malaria-related mortality and morbidity via scaling up coverage with insecticide-based interventions (1, 2) have renewed optimism that this disease can be eliminated. Two methods, which exploit key indoor resting and feeding behaviors of the most important mosquito vector species, have proven successful in several settings in Africa. The first, indoor residual spraying (IRS), was the mainstay of the World Health Organizations (WHO) malaria eradication efforts in the 1950s and 1960s and has recently re-emerged as one of the predominant malaria control tools in Africa (2). The second, currently being rolled-out on an unprecedented scale in Africa, is long-lasting insecticide-treated nets (LLINs). The major threat to the continued success of LLINs and IRS is the development of insecticide resistance in malaria vectors. Resistance is a particular threat to LLINs because there is currently only one class of insecticides, the pyrethroids, which are approved by the WHO for impregnation of bednets. In theory at least, resistance management is a more realistic option for IRS, as four classes of insecticide are available: pyrethroids, organophosphates, carbamates, and organochlorines [of which DDT, 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane, is the only organochlorine still available for malaria control]. However, although not apparent when these insecticides were first introduced, it has since become clear that these four insecticide classes share just two modes of action. The carbamates and organophosphates target the neurotransmitter acetylcholinesterase, leading to an accumulation of acetylcholine in synapses impairing nerve function (3). Pyrethroids and DDT bind to—and inhibit closure of—neuronal sodium channels, causing prolonged current, which leads to repetitive nerve firing and eventual death (4, 5). Thus, alterations in the target-site that reduce insecticide binding can cause resistance to more than one class of insecticide. Indeed, a series of mutations in the sodium channel, known as *kdr* or knockdown resistance mutations, have been conclusively linked to reduced mortality following exposure to both DDT and pyrethroids in a large number of studies (for reviews, see refs. 6 and 7). In contrast to target site resistance, very little is known about cross-resistance profiles caused by other resistance mutations. In fact it is commonly assumed that cross-resistance in a population is a result of target-site mutations, whereas other resistance mechanisms, collectively termed "metabolic resistance," are insecticide or insecticidal class-specific (8–10).

Perhaps unsurprisingly, given the focus on LLIN distribution by major donors, national malaria control programs and the media, and the increasing use of pyrethroids in IRS programs, most attention has been directed at pyrethroid resistance. Several studies have used microarray, quantitative trait loci, and association mapping-based approaches to identify detoxification genes, the expression of which is linked to pyrethroid resistance in the major malaria vectors (11–14). Multiple candidates have been identified, with cytochrome P450s from the *CYP6* class, notably *CYP6P3* and *CYP6M2*, showing the most consistent association with pyrethroid resistance (12, 15, 16). Moreover, recombinant protein expression and proteomic analysis of candidates identified through these studies have confirmed an in vitro role for some of these genes in insecticide metabolism (15, 17, 18).

In this study, in response to the resurgence in the use of DDT in sub-Saharan Africa (19), we investigate mechanisms of DDT resistance in *Anopheles gambiae s.s.* from Ghana (Fig. 1). Mosquitoes were defined as resistant if they could survive a 6-h exposure to 4% DDT and a subsequent 24-h holding period. A whole-genome microarray analysis revealed that one of the most consistently overexpressed probe sets in DDT-resistant samples encoded for *CYP6M2*, an enzyme previously implicated in pyrethroid resistance in *A. gambiae* (12, 16, 18). Functional validation confirmed that this enzyme can metabolize both DDT and pyrethroid insecticides. This finding has worrying implications for the sustained control of malaria in sub-Saharan Africa, because cross-

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Data deposition: All microarray data have been deposited in the ArrayExpress Archive (accession no A-MEXP-2196).

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resistance between insecticidal groups limits our ability to rotate active ingredients to manage resistance.

Results

Genes Differentially Expressed in DDT-Resistant Ghanaian Samples. Forty-seven microarray probes were identified as significantly differentially expressed between the three groups (Ghanaian DDT-resistant field mosquitoes, Akron Beninese controls, and Ngoussou Cameroonian controls) in the ANOVA analysis [$-\log_{10}$ false-discovery rate (FDR)-adjusted P value > 4) (Fig. 2). Post hoc pairwise t test comparisons identified 15 probes that were overexpressed in the DDT-resistant group (Table 1). Included in this group were the cytochrome P450s *CYP6M2* and *CYP9L1*, a putative aquaporin membrane transporter, and a heat-shock protein. This grouping contained a number of novel genes with no ascribed function given in the Ensembl database. The most highly and consistently (significantly) overexpressed probe was for the putative aquaporin (AGAP010326-RA). The probe was expressed 10.78-fold higher in the DDT-resistant group compared with Ngoussou and 9.04 higher compared with Akron. This gene is currently listed as "novel" in Ensembl and has "membrane transporter" listed under its Gene Ontology terms. Particularly notable is that three of the four probes for the cytochrome P450 *CYP6M2* were within the 15 probes that were overexpressed in the DDT-resistant group. The fourth was just below the significance threshold ($-\log_{10}$ FDR-adjusted P value = 3.979). There was also a remarkable consistency in the estimated fold-changes for each of the probes (Table 1).

Candidate Gene Validation. The gene *CYP6M2* was chosen for quantitative real-time PCR (qPCR) and recombinant protein-based validation. Selection was based in part on our ability to express this class of protein in recombinant *Escherichia coli* systems and previous association with insecticide, although not DDT, resistance (12, 16, 18). The other P450 significantly overexpressed in the DDT-resistant group, *CYP9L1*, was not taken forward for candidate validation because only one of the four *CYP9L1* gene probes reached significance and this probe could potentially cross-hybridize with two other P450s, AGAP012293 and AGAP012294. For qPCR of *CYP6M2*, it was not possible to design exon junction-spanning primers because of high levels of polymorphism typical of cytochrome P450 genes (20). For the *CYP6M2* plasmid standard curve, a linear relationship was recorded between concentration and C_t value, with $R^2 = 0.9998$. PCR amplification efficiency was 104%, within the acceptable range (90–105%). Qualitatively higher *CYP6M2* expression was recorded in the DDT-resistant group, 2.36-fold greater expression compared with Akron and 1.54-fold compared with the Ngoussou colony, although these values were less than half those observed in the microarray experiment (6.11-fold and 3.67-fold, respectively). This less-than-perfect concordance between qPCR and microarray datasets is a common finding in insect transcriptome studies (15, 21, 22), and possibly results from the low fold-change (23) or absolute levels of expression.

The ability of CYP6M2 to metabolize DDT was examined through HPLC analysis of in vitro metabolism of DDT in the presence of NADPH and sodium cholate. Metabolism was observed in the presence of NADPH and sodium cholate, a bile salt-type compound (Fig. 3). Sodium cholate was added to increase the solubility of DDT, a highly hydrophobic molecule (24). It was proposed that addition of this would increase the solubility and hence the availability of DDT to the P450 enzyme in the reaction. The metabolites produced by the reaction were Dicofol and DDE in a 2:1 ratio. Metabolism was enhanced by the addition of cytochrome b5. The inclusion of b5 increased the Dicofol production 10-fold, resulting in a 20:1 (Dicofol:DDE) ratio. Mean Dicofol formation from three technical replicates containing 0.01 nmol of recombinant CYP6M2 was 0.16 ± 0.02 nmol Dicofol/nmol CYP6M2/min. Additional details of the functional expression of CYP6M2 may be found in Stevenson et al. (18).

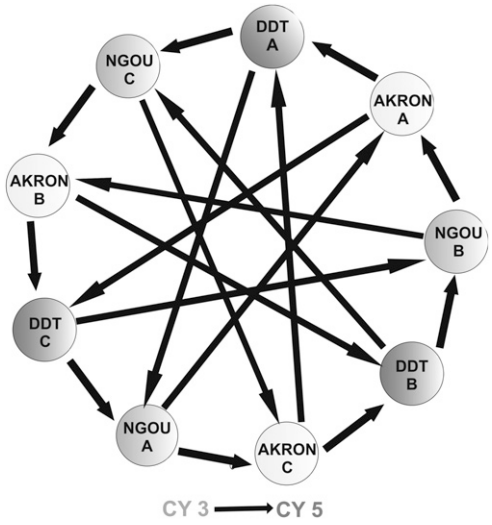

Fig. 1. Interwoven microarray experimental loop design for a comparison between DDT-resistant field-collected *A. gambiae s.s* M-forms from Ghana and two laboratory colonies of M-form *A. gambiae* originating from West Africa. The Ngoussou (NGOU) colony originates from Cameroon and is fully susceptible to DDT, but the Akron colony was colonized from Benin and displays low level DDT resistance. Each pool, indicated by a circle, represents RNA extracted from 10 female *A. gambiae s.s.* mosquitoes that were 3–5 d old. Arrows indicate individual microarrays (18 in total), with direction representing microarray Cy dye labeling.

Discussion

In this study, extremely high levels of DDT resistance were recorded in field populations of *A. gambiae* from Accra, Ghana, with a 6-h exposure to 4% DDT approximating an LT30 (exposure time required to kill 30% of the population). Although DDT resistance is widely known in Ghana (25, 26), resistance of this magnitude has not been described previously. Such high-level resistance could render DDT ineffective for IRS-based vector-control programs and understanding the mechanisms underlying resistance is important for resistance management.

We report evidence of insecticide cross-resistance ascribed to a single metabolic mechanism in *A. gambiae*. Through the application of whole-genome microarrays to study insecticide resistance in wild populations of *A. gambiae*, we were able to identify a number of differentially expressed genes in a highly DDT-resistant M-form population from Accra, Ghana, compared with more susceptible controls. One of these genes, *CYP6M2*, had previously been associated with pyrethroid resistance in *A. gambiae* (12, 16), and more recently was shown to metabolize both type I and type II pyrethroids in recombinant protein assays in vitro (18). Metabolism assays performed in the present study suggest recombinant CYP6M2 is also capable of metabolizing the organochlorine insecticide DDT, alluding to an additional role for this P450 in DDT resistance. In vivo, maximal expression of *CYP6M2* is reported in the Malpighian tubules (18), a major site of xenobiotic detoxification in insects (27–30), supporting a role in insecticide clearance.

Intriguingly, DDT metabolism was dependent upon the presence of the bile acid, sodium cholate. It is not unusual for P450s to exhibit heterotropic cooperativity, because the P450 active-site can often accommodate more than one compound (31), and this is a common mechanism of in vivo drug–drug interactions in humans (32). The pharmacokinetics of DDT and CYP6M2 interactions in mosquitoes is likely to be very complex, and

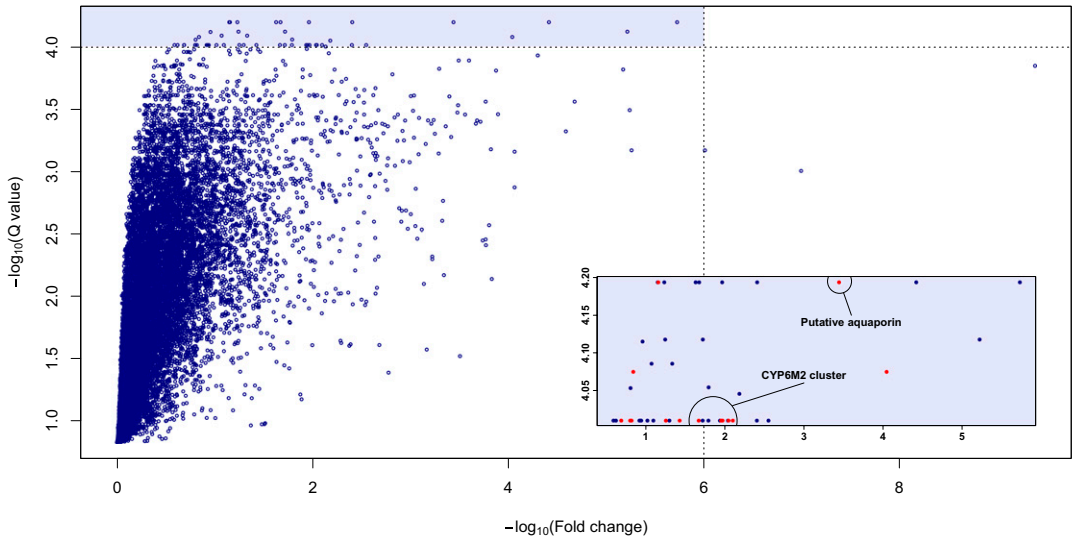

Fig. 2. Volcano plot showing the reproducibility of differences in gene expression between the three groups (Ghanaian DDT-resistant *A. gambiae s.s.* from the field and the Akron and Ngoussou control colonies). The figure differs from a conventional volcano plot as there are three treatment groups as opposed to the more usual two. Q values, FDR-adjusted *P* values ($-\log_{10}$), are shown along with Log_2 fold-changes. A Q value of 4 ($q < 0.0001$) is shown as the significant test level. A selection of genes from Table 1 found to be significantly overexpressed in the DDT-resistant group are indicated.

further investigation of CYP cooperativity is warranted. In mammals, sodium cholate is synthesized in the liver from cholesterol by cytochrome P450s and facilitates digestion of triacylglycerols (33). It is thought that insects lack bile salts (34) and it is currently unknown if *A. gambiae* deploys another mechanism to increase lipid solubility.

CYP6M2 is the second *A. gambiae* P450 found capable of metabolising DDT. A previous study found that CYP6Z1, expressed using insect cell lines and a baculovirus system, was able to metabolize DDT in vitro (17). In the former study, metabolism was not found to be sodium cholate-dependent. This finding may reflect differences in the dynamics of membrane interactions and substrate uptake from the lipid bilayer in insect cells (17) and *E. coli* (present study), which is currently under investigation.

CYP6M2 is unique as a mosquito P450 shown to metabolize two different classes of insecticide. In only one previous instance has a single P450 been linked to resistance to more than one insecticidal compound. The *Drosophila melanogaster* P450, *CYP6g1*, is associated with resistance to neonicotinoids, the organophosphate malathion and organochlorines DDT and methoxychlor (35–38). Cross-resistance is arguably an unexpectedly infrequent observation, given the broad substrate specificity of P450s to, for example, plant toxins (39, 40).

A number of additional genes were also identified as up-regulated in the Ghanaian DDT-resistant mosquitoes, including putative membrane transporters and heat-shock proteins. For all but the P450 enzymes, it has only been possible to speculate about their possible role in the insecticide-resistance phenotype. However, these data, and similar studies in *Drosophila* (41), can be seen to not only reveal the promise of whole-genome analysis for insecticide resistance studies but, given the incrimination of *CYP6M2*, validate the earlier candidate-gene approach (12, 14–16).

Putative Aquaporin Membrane Transporter. A putative membrane transporter, AGAP010326-RA, was the most significantly ($-\log_{10}$ FDR-adjusted $P = 4.194$) and highly overexpressed (9.04- to 10.78-fold) gene in the DDT-resistant group. The closest protein orthologs are putative aquaporins from mosquitoes *Aedes aegypti* (AAEL005001, 55% amino acid identify), *Culex quinquefasciatus* (CPIJ009225, 42% amino acid identity), and the louse *Pediculus humanus* (PHUM474700, 38% amino acid identity). Aquaporins are membrane proteins involved in the movement of water and other solutes across biological membranes, and in insects are often associated with the excretory/osmoregulatory Malpighian tubule system (42, 43).

The Malpighian tubules have been shown to be a focus of P450-mediated DDT metabolism in *Drosophila* (44). *CYP6g1* is enriched 9.4-fold in *Drosophila* Malpighian tubules (29) and site-directed RNAi in the Malpighian tubules caused a significant increase in DDT susceptibility (44). The Malpighian tubules are believed to be the primary route of DDT excretion in vivo (45) and the potential upregulation of *CYP6M2* and a putative aquaporin in the mosquito Malpighian tubules could be components of a coordinated evolutionary response related to efflux/excretion of DDT and its metabolites (DDE or Dicofol).

Conclusion

From a public health perspective it is a major concern that overexpression of a single P450 has been linked to resistance to two of the four insecticide groups licensed for malaria control. Support for this resistance association is provided by the in vitro data, which show that CYP6M2 is able to metabolize both DDT and class I and II pyrethroids (18). DDT and pyrethroids share a common target-site and are therefore suboptimal for use in combination; nevertheless, they are being deployed simultaneously in a number of areas in sub-Saharan Africa (46). The presence of cross-resistance confounds resistance-management strategies. We are fortunate that the apparent cross-resistance is between insecticides that share a target-site. Cross-resistance between active ingredients with different target-sites will be a far less tractable problem.

Materials and Methods

Collection and DDT Phenotyping of Mosquitoes from Ghana. Mosquito larval collections were performed between the May 9 and 30, 2008. Eight breeding sites within an area of 30 km^2 in the Greater Accra region were located by searching for water bodies in areas known to harbor *A. gambiae* (47). Larvae were reared to adults at the Biotechnology and Nuclear Agriculture Research Institute, Accra. Pupae were picked daily and placed into plastic cages. To standardize the age of adults for testing, pupae harvested from a maximum of 3 d were placed in a single cage. Upon emergence, adults of both sexes were maintained on a 10% (wt/vol) sugar solution until 3–5 d posteclosion. Insecticide selections were performed on nonblood-fed females mosquitoes

Table 1. Microarray probes which were significantly (FDR-corrected $P < 0.0001$) overexpressed in the Ghanaian DDT-resistant group of *A. gambiae s.s* compared with both Akron and Ngoussou colonies

Description			Fold-change (−log Q value)			Overall *F* test		
Probe	Gene	Orthologs	Akron v Ngoussou	Akron v Ghana	Ngoussou v Ghana	Fold-change	logQ	Order
CUST_3425_PI4	AGAP001827-RA	Hypoxia up-regulated/ hsp 70 - c.e. [T14G8.3, T24H7.2, 29–32%]	0.80 (2.83)	1.74 (3.58)	2.16 (3.67)	2.23	4.194	G > A > N
CUST_8992_PI4	AGAP010326-RA	Aquaporin - a.a. [AAEL005001, 48%], p.h. [PHUM474700, 33%]	0.84 (1.13)	9.04 (3.67)	10.79 (3.67)	10.85	4.194	G>>A > N
CUST_935_PI42	AGAP005501-RA	Alcohol dehydrogenase (putative) - p.h. [PHUM184020, 48%]	0.24 (3.33)	2.65 (2.98)	11.10 (3.67)	16.46	4.075	G>>A > N
CUST_1791_PI4	AGAP006276-RA	No information available in VectorBase	0.95 (1.22)	1.69 (3.58)	1.79 (3.58)	1.79	4.075	G > A > N
CUST_11496_PI	AGAP008212-RA	CYP6M2	1.66 (2.48)	5.88 (3.58)	3.55 (3.43)	3.91	4.011	G > N > A
CUST_7357_PI4	AGAP001541-RA	No ortholog. GO terms-Meprin and TRAF homology domain-containing protein	2.66 (3.55)	3.04 (3.58)	1.14 (1.34)	2.69	4.011	G > N > A
CUST_8742_PI4	AGAP000385-RB	Lethal (1) G0193 (molecular function unknown) - d.m. [FBgn0027280, 37%]	1.74 (3.48)	1.98 (3.58)	1.14 (1.93)	1.76	4.011	G > N > A
CUST_8744_PI4	AGAP000385-RC	Lethal (1) G0193 (molecular function unknown) - d.m. [FBgn0027280, 37%]	1.71 (3.47)	1.96 (3.58)	1.15 (1.97)	1.74	4.011	G > N > A
DETOX_510_PI4	AGAP012295-RA	CYP9L1	0.39 (3.55)	1.05 (0.71)	2.66 (3.57)	3.86	4.011	G > A > N
CUST_3447_PI4	AGAP001874-RC	Rap 1 (ras GTPase) "cellular switch for signal transduction" - a.a. [AAEL009377, 98%], p.h. [PHUM226110, 93%]	1.55 (3.33)	1.87 (3.58)	1.20 (2.44)	1.61	4.011	G > N > A
CUST_8860_PI4	AGAP000461-RA	Type II transmembrane protein - c.q. [CPIJ011363, 69%], p.h. [PHUM617500, 51%]	0.45 (3.55)	1.03 (0.61)	2.30 (3.57)	3.18	4.011	G > A > N
CUST_5671_PI4	AGAP013468-RA	Adam (a disintegrin and metalloprotease domain), c.q. [CPIJ012679, 45%], a.a. [AAEL005992, 45%]	0.87 (1.66)	2.04 (3.46)	2.35 (3.58)	2.38	4.011	G > A > N
CUST_8745_PI4	AGAP000385-RD	Lethal (1) G0193 (molecular function unknown) - d.m. [FBgn0027280, 37%]	1.71 (3.40)	1.97 (3.58)	1.15 (1.91)	1.74	4.011	G > N > A
DETOX_441_PI4	AGAP008212-RA	CYP6M2	1.64 (2.29)	6.19 (3.58)	3.77 (3.33)	4.12	4.011	G>>N > A
DETOX_439_PI4	AGAP008212-RA	CYP6M2	1.74 (2.38)	6.68 (3.58)	3.83 (3.31)	4.28	4.011	G>>N > A

Description indicates gene name if defined in Ensembl or Gene Ontology (GO) terms and orthologs, where genes are listed as "novel" in Ensembl. [%] Percentage identity of orthologs in VectorBase (http://www.vectorbase.org/), a.a., *Aedes aegypti*; c.q., *Culex quinquefaciatus*; d.m. *Drosophila melanogaster*; p.h., *Pediculus humanis*; c.e., *Caenorhabditis elegans*.

aged 3–5 d. Mosquitoes were exposed to 4% (wt/vol) DDT following WHO protocols (48). Mortality was assessed 24 h after the end of the exposure period. Initial experiments showed that the level of DDT resistance was extremely high and that it would not be possible to produce a full-time response curve, as performed previously (15). A 6-h exposure was found to approximate an LT30. Mosquitoes that died within 24 h after the 6-h exposure were placed individually on silica gel for preservation. Resistant females, alive after 24 h, were chilled at 4 °C to prevent escape during transfer to tubes. A hind leg was removed using forceps and the body of the mosquito was then submerged in RNA preservative solution, RNAlater (Ambion) and treated according to the manufacturer's instructions, with samples chilled overnight at 4 °C to allow the solution to penetrate the material before transfer to a −20 °C freezer.

For each female, species, molecular form identification, and target-site mutation characterization was conducted on the amputated hind leg. The legs were transferred to a 96-well plate and DNA extracted using the DNeasy Blood and Tissue kit (Qiagen). The standard protocols to identify *A. gambiae s.l.* species and molecular form were used (49, 50). Both of the molecular forms (subspecies) of *A. gambiae*, termed the M and S forms, are found in the study area (51). The *L1014F* and *L1014S kdr* mutations in the voltage-gated sodium channel, which confer target-site resistance to DDT and pyrethroids, were screened for using a Taqman assay (52).

Microarray Experimental Design. We selected *A. gambiae s.s.* M-form mosquitoes, which were the larger proportion of those phenotyped (63%). Wild-caught individuals that were selected for analysis were *wildtype/L1014F* heterozygotes at the *kdr* locus, the predominant genotype in the M-form (76%). Given the high levels of DDT resistance in the area, it was not possible to obtain sympatric susceptible samples to use as a comparator as has been done in previous microarray studies (15). We therefore selected two West African M-form colonies that displayed no or very low levels of DDT resistance. The Ngoussou colony originates in Cameroon and is a fully DDT-susceptible *A. gambiae s.s.* colony with no known *kdr* mutations. The Akron colony from Benin has low level DDT resistance ($LT_{50} \sim 1$ h) and some *kdr* mutations, but at lower frequencies than the Ghanaian population [frequency (*kdr*): Ghanaian M-form 0.58, Akron M-form colony 0.29]. The Akron colony was established in June 2008 at around the same time that Yadouleton et al. were assessing DDT susceptibility in the same location (53). In the latter study far higher levels of DDT resistance were observed (15% mortality following a WHO tube assay). We suggest that the apparent loss of DDT resistance in the laboratory colony is a result of a marked founding effect and relaxation of insecticidal selection pressure. This use of two control groups, and the stringent ANOVA analysis detailed below, limits the influence of potential confounders, such as colonization effects, differing rearing environments and, in this instance, geographical origin. Colony specimens for expression analyses were prepared in

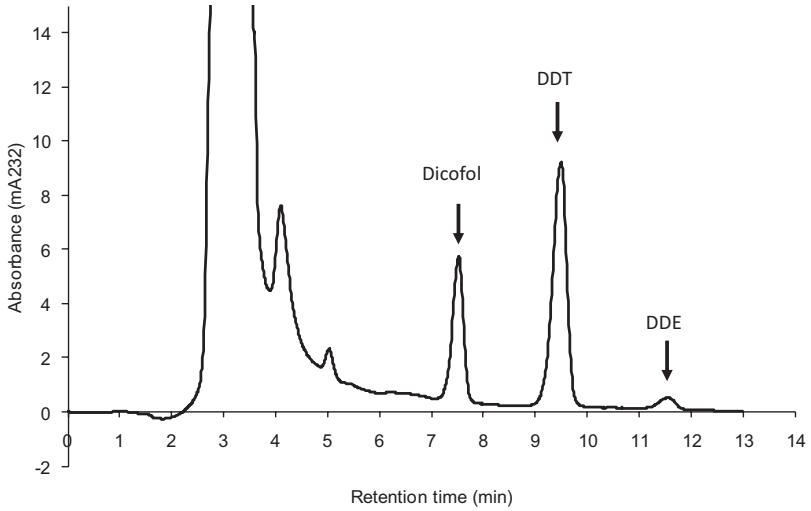

Fig. 3. HPLC trace showing CYP6M2 mediated DDT metabolism. HPLC chromatogram of reaction products of DDT incubated with CYP6M2 membranes in the presence of 1 mM cholate and NADPH. Retention times of suspected metabolites (DDE and dicofol) were confirmed through HPLC. In reactions containing cholate but minus NADPH, no dicofol or DDE was detectable (figure omitted for clarity).

exactly the same manner as the field-caught test subjects except that they were exposed to control, rather than insecticide-treated papers. To date, sublethal exposure to insecticide is not thought to result in wide-scale gene induction (54–56). Nevertheless, as there is some evidence for short-lived induction (57), the mosquitoes are held for 24 h postexposure so that any genes that are differentially expressed between groups most likely reflect constitutive rather than induced changes. An interwoven loop design, using three RNA pools from field-collected material and each colony control, was selected for between group comparisons because this experimental design is both more efficient and provides greater power compared with the standard experimental design (58). The experimental design is shown in Fig. 1.

Microarray Design: "AGAM_15K." For this study, a new Agilent 8 × 15 K *A. gambiae s.s.* microarray design was created. The array was designed in eArray (https://earray.chem.agilent.com/earray/). Full details of the array design are given in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), but in brief the array contains 14,071 probes for the 12,604 *A. gambiae s.s.* genes identified in the Ensembl P3.5 annotation (September 2009). For genes with alternative splice variants, separate probes target individual variants. An additional three unique probes per gene were designed for the 281 insecticide-resistance candidate gene sequences from the *A. gambiae* detox array (14); thus, detoxification candidates are covered by four separate probes.

RNA Extractions. Total RNA extractions were performed on pools of five female mosquitoes grouped by population. All RNA extractions were performed using TRI Reagent (Ambion) and DNase treated (TURBO- DNase-free, Ambion). Because of between-pool variability observed in preliminary studies, two pools of RNA were combined based on similar concentrations and 2100 Bioanalyzer (Agilent Technologies) profiles to create single RNA pools comprised of 10 female mosquitoes. The quality and quantity of the RNA in the combined pools were assessed using a Bioanalyzer and a NanoDrop spectrophotometer (NanoDrop Technologies), respectively (Table S1).

Microarray Hybridization and Analysis. The RNA pools selected for microarray analysis were labeled separately with Cy3 and Cy5 dyes using the Low Input Quick Amp Labeling Kit (Agilent Technologies). Labeled RNA quantity and quality were assessed using a NanoDrop spectrophotometer and a Bioanalyzer (Table S2). All samples passed Agilent recommendations for yield greater than 825 ng and specific activity greater than 6.0 pmol of cynanine (Cy) per microgram of cRNA. Array hybridization, washing, scanning, and feature extraction were performed according to the manufacturer's recommendations. All arrays passed the Agilent quality control thresholds (QC score ≥ 10). Microarray normalization was performed in the statistical program R using Limma 3.2.3 (59), as described previously (12). Analysis of normalized signal intensities was performed using the MAANOVA package in R (60). The analytical script is detailed in Dataset S1. In brief, because there were three treatment groups, an ANOVA *F*-test approach was applied. The significance level for the FDR-corrected data (61) was set at $\log_{10}$(Q value) > 4 (q < 0.0001). Within this subset of significantly differentially expressed probes, those that were significantly overexpressed in the DDT-resistance group were identified by examining expression patterns in all pair-wise comparisons.

Candidate Gene Validation by qPCR. Real-time quantitative PCR primers were designed for the resistance associated candidate *CYP6M2* using sequence data from Ghanaian field-collected mosquitoes. cDNA pools were produced from RNA samples used in the microarray experiment via reverse-transcription using SuperScript III (Invitrogen) primed with an oligo$(dT)_{20}$ primer following the manufacturer's guidelines. Ribosomal S7 (GenBank: L20837) and gene-specific plasmids were used to standardize the data (Table S3).

Functional Analysis of DDT Metabolism by Recombinant *CYP6M2*. Recombinant protein expression of one of the major resistance-associated candidates, the P450 CYP6M2, followed published protocols (15, 18). *CYP6M2* from *A. gambiae s.s.*, with a bacterial ompA+2 leader sequence, was coexpressed in the DH5α cell line along with *A. gambiae s.s.* cytochrome P450 reductase and membranes prepared according to previous protocols (18). CYP6M2 concentration was assessed from whole-cell and membrane fractions via hemoprotein content using $Fe2^{+}$-CO^{-} vs. $Fe2^{+}$ difference spectroscopy (62), before use in DDT metabolism assays.

The DDT metabolism assays were performed in 100-μL reaction volumes containing 0.2 M Tris-HCl at pH 7.4, 1 mM glucose-6-phosphate, 0.25 mM $MgCl_2$, 0.1 mM $NADP^{+}$, 1 unit/mL glucose-6-phosphate dehydrogenase (G6PDH), 10 μM DDT (dissolved in ethanol), 0.1 μM CYP6M2, 0.8 μM cytochrome b_5, and sodium cholate (Sigma) at an optimized concentration (1 mM), ensuring an overall 2% (vol/vol) ethanol content. Controls were performed minus $NADP^{+}$ and G6PDH. Each reaction was performed in triplicate and incubated at 30 °C with 1,200 rpm shaking for 60 min. Reactions were stopped by addition of 100 μL acetonitrile, followed by a further incubation for 20 min (30 °C, 1,200 rpm) to ensure all reaction products were in solution, and then centrifuged for 5 min at 20,000 × *g* to remove the membranes. Supernatant (150 μL) was transferred to glass HPLC vials (Chromacol) before the presence of DDT and potential metabolites was assessed via reverse-phase HPLC analysis at an absorbance wavelength of 232 nM (Chromeleon; Dionex). Samples were injected at a volume of 100 μL into an isocratic mobile phase of 90% methanol and 10% water with a flow rate of 1 mL/min and substrate peaks separated with a 250 mm C18 column (Acclaim 120; Dionex) at 23 °C. DDT eluted at 9 min 31 s with metabolites Dicofol and DDE eluting at 7 min 31 s and 11 min 32 s, respectively.

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