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A *cis*-regulatory sequence driving metabolic insecticide resistance in mosquitoes: functional characterisation and signatures of selection

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Abstract

Although cytochrome P450 (CYP450) enzymes are frequently up-regulated in mosquitoes resistant to insecticides, no regulatory motifs driving these expression differences with relevance to wild populations have been identified. Transposable elements (TEs) are often enriched upstream of those CYP450s involved in insecticide resistance, leading to the assumption that they contribute regulatory motifs that directly underlie the resistance phenotype. A partial *CuRE1* (*Cu*lex *R*epetitive *E*lement 1) transposable element is found directly upstream of *CYP9M10*, a cytochrome P450 implicated previously in larval resistance to permethrin in the ISOP450 strain of *Cx. quinquefasciatus*, but is absent from the equivalent genomic region of a susceptible strain. *Via* expression of CYP9M10 in *E.coli* we have now demonstrated time- and NADPH-dependant permethrin metabolism, prerequisites for confirmation of a role in metabolic resistance, and through qPCR shown that *CYP9M10* is >20-fold over-expressed in ISOP450 compared to a susceptible strain. In a fluorescent reporter assay the region upstream of *CYP9M10* from ISOP450 drove 10x expression compared to the equivalent region (lacking *CuRE1*) from the susceptible strain. Close correspondence with the gene expression fold-change implicates the upstream region including *CuRE1* as a *cis*-regulatory element involved in resistance. Only a single *CuRE1* bearing allele, identical to the *CuRE1* bearing allele in the resistant strain, is found throughout Sub-Saharan Africa, in contrast to the diversity encountered in non-*CuRE1* alleles. This suggests a single origin and subsequent spread due to selective advantage. *CuRE1* is detectable using a simple diagnostic. When applied to *Cx. quinquefasciatus* larvae from Ghana we have demonstrated a significant association with permethrin resistance in multiple field sites (mean Odds Ratio = 3.86) suggesting this marker has relevance to natural populations of vector mosquitoes. However, when *CuRE1* was excised from the allele used in the reporter assay through fusion PCR, expression was unaffected, indicating that the TE has no direct role in resistance and hence that *CuRE1* is acting only as a marker of an as yet unidentified regulatory motif in the association analysis. This suggests that a re-evaluation of the assumption that TEs contribute regulatory motifs involved in gene expression may be necessary.

1. Introduction

The control of a number of pernicious mosquito-borne diseases such as malaria, lymphatic filariasis and dengue is reliant upon the application of insecticides. Insecticide exposure is a potent selective force and resistance can emerge over a relatively short period of time, threatening the efficacy of control programs. Resistance commonly arises as a consequence of mutations in the neuronal target sites of the insecticides or through copy number variation, elevated expression of, or allelic variants in, metabolic detoxification enzymes (Ranson et al., 2011). Resistance mutations in target-sites are commonly non-synonymous changes which can be identified relatively readily and screened for in natural populations. Indeed, for the African malaria vector *Anopheles gambiae*, screening for mutations in the voltage gated sodium channel that confer resistance to DDT and pyrethroid insecticides, e.g. the *1014F* and *1014S* mutations commonly referred to as knock down resistance or *kdr* mutations, has become a routine component of many disease vector control campaigns (e.g. Chanda et al., 2011; Himeidan et al., 2011; Mathias et al., 2011; Yewhalaw et al., 2011). Given that many resistance mutations are thought to be recessive in expression, development of DNA diagnostics enables disease and agricultural control programmes to identify resistance mechanisms when they are at low frequency and take ameliorating action before they cause economic or health impacts (Hemingway, 2009; Kelly-Hope et al., 2008). As shown in a number of recent studies, resistance alleles can rise towards fixation very rapidly (García et al., 2009; Lynd et al., 2010; Mathias et al., 2011), thus advanced warning of segregation of low-frequency resistance alleles in a population could be immensely beneficial.

In whole genome microarray studies of pyrethroid resistant mosquitoes, members of the cytochrome P450 class of metabolic enzymes are frequently up-regulated (Djouaka et al., 2008; Mitchell et al., 2012; Muller et al., 2008; Wondji et al., 2009) suggestive of a predominant role in the resistance phenotype. Confirmatory evidence of a direct role in resistance must come from *in vitro* metabolism of insecticides with heterologously expressed CYP450, and for a limited number of

CYP450s this evidence has been attained (Chiu et al., 2008; Mitchell et al., 2012; Muller et al., 2008; Stevenson et al., 2011). However, whilst gene expression differences coupled with *in vitro* metabolic activities have confirmed the roles of a number of CYP450s in metabolic resistance to insecticides, to date, the causal mutations that result in this over-expression have remained elusive.

Motifs in the region directly upstream of genes responsible for the binding of transcription factors (*cis*-elements), and particularly those in the promoter region, are likely candidate regions for resistance associated variants. However, identification of *cis*-regulatory elements poses a particular challenge. Whilst some progress has been made in our knowledge of these (Lenhard et al., 2012; Wittkopp and Kalay, 2012), and conserved motifs upstream of genes are identifiable in mosquitoes which may represent *cis*-regulatory elements (Sieglaff et al., 2009), validated statistical methods for identification of motifs in sequence data are lacking for many species (Down et al., 2007; Saminadin-Peter et al., 2012). Transposable elements (TEs) are ubiquitous features of mosquito genomes (Boulesteix and Biemont, 2005; Fernandez-Medina et al., 2011; Tu and Coates, 2004) with some 29% of the assembled *Culex quinquefasciatus* genome composed of TEs (Arensburger et al., 2010). TEs can contribute regulatory motifs affecting gene expression when inserted upstream of genes (Ganko et al., 2003; Jordan et al., 2003; Thornburg et al., 2006; van de Lagemaat et al., 2003). TEs with roles in regulating expression levels would therefore represent readily identifiable regulatory elements.

Numerous examples exist of TEs located in close proximity to genes involved in metabolic resistance in insects (Chen and Li, 2007; Chen and Li, 2008; Li et al., 2007). Perhaps the best described example of a TE inserted close to a functional CYP450 is *CYP6g1* in *Drosophila melanogaster* which confers resistance to DDT and for which the allele containing the TE now has a worldwide distribution (Brookfield, 2004; Catania et al., 2004; Daborn et al., 2002; Schlenke and Begun, 2004; Schmidt et al., 2010). In the CYP450 family generally, transposable elements are over-represented in proximity to insect CYP450s involved in metabolic detoxification whilst being largely absent from CYP450s involved in essential processes such as ecdysone biosynthesis and developmental regulation (Chen

and Li, 2007). This suggests not only that TEs are selectively retained upstream of P450s putatively involved in insecticide resistance but that they are likely to have a functional role, perhaps contributing regulatory motifs that control or contribute to the resistance phenotype.

Culex quinquefasciatus has a widespread tropical and sub-tropical distribution, vectors West Nile virus and lymphatic filariasis, and is a major biting nuisance in the urban tropics. The JPAL strain of *Cx. quinquefasciatus* was established from Saudi Arabia (Amin and Hemingway, 1989) and exhibits high resistance in the aquatic larval stage to permethrin (a class I pyrethroid) but relatively low resistance to class II pyrethroids such as deltamethrin (resistance ratios of 2,500 and 56 respectively, compared to the susceptible Ogasawara lab strain (Weerasinghe et al., 2001)). In a series of experiments Tomita and colleagues implicated mutations in the sodium channel (*L1014F*), the target-site of permethrin, and increased expression of cytochrome P450s as the likely causes of the permethrin resistant phenotype (Itokawa et al., 2011; Itokawa et al., 2010; Komagata et al., 2010) in JPAL. Using a custom microarray incorporating probes for 197 genes including 62 CYP450s, three genes, *CYP9M10, CYP4H34* and *CYP6Z10,* were detected as up-regulated (39.2, 5.6 and 2.6 fold respectively) in JPAL relative to the Ogasawara strain (Komagata et al., 2010). Two of the three genes, *CYP9M10* and *CYP4H34*, showed much higher transcription in larvae, implicating these as potential mediators of the larval resistance phenotype.

However these studies were not able to determine the relative importance of the target site (*L1014F*) mutation and the CYP450 over-expression in conferring the resistance phenotype. To address this, Hardstone et al. (2007) developed the ISOP450 strain of *Cx. quinquefasciatus* through back-crossing JPAL to the standard susceptible S-LAB strain and selecting larvae at the L4 stage with permethrin, so embedding the pyrethroid resistance locus or loci in the S-LAB genetic background. *Via* crossing and selection, *kdr*-mediated resistance was removed such that permethrin resistance in ISOP450 is mediated solely by metabolic resistance. Only *CYP9M10* is highly over-expressed in ISOP450 relative to S-LAB (Hardstone et al., 2010) indicating that this locus, and not *CYP4H34*,

underlies the resistance in this strain and additionally suggesting it may also be the causal mechanism in the parent JPAL strain.

Crosses between the JPAL and Ogasawara strains, followed by allele-specific qPCR were used to infer that the over expression of *CYP9M10* in JPAL was under the control of a *cis*-acting element (Itokawa et al., 2010). A likely regulatory element is *CuRE1* (*Culex* Repetitive Element 1) a Minitature Inverted-repeat Transposable Element (MITE) which is found upstream of *CYP9M10* in the JPAL but not the susceptible Ogasawara strain (Itokawa et al., 2010). *CYP9M10* is also duplicated in JPAL (Itokawa et al., 2010) with the duplicate gene copies (*CYP9M10* and *CYP9M10v2*) having identical amino acid sequences and sharing the same upstream sequence, including the *CuRE1* element, up to 1.1kb 5' of *CYP9M10*. However, this duplication alone is insufficient to explain the resistance patterns. Whether *CuRE1* is an important marker for permethrin resistance in wild populations or contributes regulatory motifs resulting in gene expression differences has not yet been answered.

In this study we demonstrate the ability of heterologously-expressed CYP9M10 to metabolise permethrin and confirm the findings of Hardstone et al. (2010) that *CYP9M10* is up-regulated in the ISOP450 strain. Using a reporter assay we demonstrate that the genomic region upstream of *CYP9M10* from the resistant strain contains regulatory motifs, but, crucially, that the *CuRE1* element is not the origin of these. This suggests that a re-evaluation of the assumption that TEs inserted upstream of CYP450s consistently contribute functional motifs is necessary. Additionally, we detect evidence of strong selection on this genomic region, the consequence of which is that whilst *CuRE1* is not causal, it is in tight linkage disequilibrium (LD) with the causal motif and can hence act as a marker of metabolic resistance in wild-caught mosquitoes.

2. Materials and Methods

2.1 Strains of Cx. quinquefasciatus *used*

The ISOP450 strain of *Culex quinquefasciatus* was supplied by Dr J. Scott (Cornell University) and exhibits larval resistance to permethrin (Hardstone et al., 2007). The CqSF strain from Recife, Brazil was supplied by Dr C. Fontes (Centro de Pesquisa Aggeu Magalhães, Recife, Brazil) and is classified as susceptible. To confirm the CqSF phenotype we performed standard larval bioassays following WHO protocols (W.H.O., 2005). Briefly, batches of 25 larvae were exposed to permethrin concentrations (ranging from 0.0001 to 10ppm) in 100ml water and mortality determined after 24 hrs. 'Dead' individuals were those unable to mount an escape response to capture with forceps. Log-concentration *vs* mortality curves were generated from these bioassay data and LC_{50} calculated in R (R Development Core Team, 2011).

2.2 RNA isolation and qPCR

RNA was isolated from pools of 30 L4 larvae using either the Picopure kit (Arcturus) or the RNAqueous4PCR kit (Ambion) and cDNA produced from 2.5µg total RNA using Superscript III (Invitrogen) and an oligo dT_{20} primer. The cDNA was cleaned using a Qiagen PCR purification kit prior to use in qPCR experiments. A five-fold dilution series (1 to 1/15,625) of both CqSF and ISOP450 cDNAs was used to produce standard curves. Primers for qPCR of *CYP9M10* were Cx_9M10qPCR-1 and Cx_9M10qPCR-4, and for the normalising 40S ribosomal protein S7 (CPIJ006763) Cx_S7qPCR-1 and Cx_S7qPCR-2 (see supplementary material for these and all subsequent primer sequences). Both sets of primers span an exon-exon junction. Primers for *CYP4H34* were CYP4H34-1 and CYP4H34-2 of Komagata et al. (2010). Since *CYP4H34* is intron-less these do not span an exon-exon junction.

qPCR reactions were conducted in triplicate in 20µl volumes containing 1x Agilent Brilliant III SYBR qPCR mastermix, 300nM each primer (with the exception of primer Cx_9M10qPCR-4 which was used at 600nM following optimisation) and 1µl template RNA (1/625 dilution) on an Agilent MX3005 qPCR

system with cycling conditions of 3min at 95°C and 40 cycles of 10s at 95°C and 10s at 60°C. The $\Delta\Delta C_t$ method was used for calculation of fold changes (Livak and Schmittgen, 2001).

2.3 Cloning and expression of P450s

Full length cDNAs of *CYP9M10* and *CYP4H34* from both resistant (ISOP450) and susceptible (CqSF) strains were amplified from cDNA by RT-PCR using primer pairs CYP9M10expFOR1 and CYP9M10expREV1 or CYP4H34expFOR1 and CYP4H34expREV1. Purified products were cloned using the cloneJET PCR cloning system (Fermentas) and sequenced. Full length sequences (Accession numbers JQ001925 and JQ001926 for CqSF and ISOP450 *CYP9M10* sequences respectively and JQ001927 and JQ001928 for ISOP450 and CqSF *CYP4H34* sequences) are provided in the supplementary material.

For expression, a bacterial ompA leader sequence was fused to both *CYP9M10* and *CYP4H34* and ligated into the expression vector pCW-ori+ plasmid, pB13 (Pritchard et al., 1998) to produce pB13::CYP9M10 and pB13::CYP4H34, as described previously (McLaughlin et al., 2008; Pritchard et al., 1997). Further details are provided in the supplementary material.

Competent JM109 cells (Promega) were co-transformed with pB13::CYP9M10 or pB13::CYP4H34 and pACYC-AgCPR (McLaughlin et al., 2008) for co-expression of P450 and its redox partner *An. gambiae* cytochrome P450 reductase (CPR). Expression, membrane isolation and determination of P450 content was carried out as described previously (McLaughlin et al., 2008; Muller et al., 2008; Stevenson et al., 2011) – see supplementary material. P450 activity and concentration was estimated through CO difference spectrum (Omura and Sato, 1964), total protein through Bradford assay with BSA standards and CPR by cytochrome *c* reduction (Strobel et al., 1978).

2.4 Metabolism assays

Metabolism assays were conducted by incubating the recombinant P450 complex with two pyrethroid insecticides. Permethrin (46% cis:52% *trans*) and deltamethrin (Greyhound

Chromatography and Allied Chemicals, Birkenhead, U.K.) were dissolved in 20% ethanol before addition to the reaction with a final ethanol content of 2% in metabolism reactions. Reaction conditions were 100µl 0.2M Tris-HCl pH 7.4, 1mM glucose-6-phosphate, 0.25mM $MgCl_2$, 0.1mM NADP+, 1U/ml glucose-6-phosphate dehydrogenase (G6PDH), 0.1µM CYP9M10 or CYP4H34, 0.8µM b5 and 20µM deltamethrin or permethrin. A negative NADPH control omitted NADP+ and G6PDH. Reactions were incubated at 30°C with shaking (1200rpm) with membranes kept separate from reaction buffer for 5min before the reaction was initiated by vortexing. A tenth of a ml of acetonitrile containing 20µM bifenthrin (as internal HPLC standard) was added to stop reactions. Following shaking (1200rpm) for 20min at 30°C in order to ensure dissolution of all pyrethroids, samples were centrifuged at 20,000g for 5min and the substrate transferred to HPLC vials then analysed by reverse-phase HPLC with a monitoring absorbance of 232nm (Chromeleon, Dionex). 100µl was loaded into an isocratic mobile phase of 90% acetonitrile and 10% water with a flow-rate of 1ml/min and substrate peaks separated with a 250mm C18 column (Acclaim 120, Dionex) at 23°C. Deltamethrin eluted at ≈11.5min and *cis*- and *trans*- permethrin at 15.2 and 18.5 min respectively. Time course metabolism assays of permethrin metabolism using CqSF CYP9M10 membranes were conducted as above but for 0, 5, 10, 20, 30 and 60 minutes before reactions were quenched.

2.5 Luciferase reporter assay of **CYP9M10** *promoter region*

The region immediately 5' of *CYP9M10* from both ISOP450 and CqSF strains were amplified using primers 9M10UP5 and 9M10UP8. The closest gene 5' of *CYP9M10* is the CYP450 *CPIJ014219* (encoded on the opposite strand) and the primer 9M10UP5 sequence is situated within *CPIJ014219*. Primer 9M10UP8 is sited within the 5' UTR of *CYP9M10*. These primers gave a ≈ 1.5kb region from ISOP450 and 834bp from CqSF. Because the breakpoint 5' of the duplicated *CYP9M10* in JPAL, the parent strain of ISOP450, is 1.1kb upstream, the amplified region is from *CYP9M10* and not *CYP9M10v2* (see Fig. 4 of Itokawa et al. 2010). Products were cloned into pJET1.2 (Fermentas) and sequenced. From these sequences two primer pairs 9M10UP5-KPNI and 9M10UP6-KPNI were

designed sharing a common reverse primer 9M10UP8-HINDIII terminating 3bp from the start of the *CYP9M10* coding sequence. Primers incorporated either *Hin*dIII or *Kpn*I to facilitate cloning into the pGL3-Basic reporter vector. Products (1504bp and 1315bp for ISOP450, and 834bp and 647bp for CqSF for 9M10UP5-KPNI-9M10UP8-HINDIII and 9M10UP6-KPNI-9M10UP8-HINDIII respectively) were amplified using Phusion high fidelity polymerase (Fermentas) and cloned into PJET1.2. Following excision with *Hin*dIII and *Kpn*I, these were ligated into pGL3-Basic (Promega) and transformed into XL1-Blue (Agilent). To remove the *CuRE1* element whilst retaining all additional differences between the susceptible and resistant alleles, fusion PCR with primers 9M10UP5-KPNI or 9M10UP6-KPNI and Cure1_fusion_3, and 9M10UP8-HINDIII and Cure1_fusion_4 was undertaken. Products of both PCRs were diluted 1/100 and 1µl used in PCR with 9M10UP5-KPNI or 9M10UP6-KPNI and 9M10UP8-HINDIII to knit the fragments together. Products were cloned into PJET1.2 excised with *Hin*dIII and *Kpn*I, ligated into pGL3-Basic and sequenced. Plasmids were extracted with GeneJet plasmid miniprep kit (Fermentas) and concentrations adjusted to 100ng/µl. Dual luciferase assays were undertaken using *An. gambiae* cell line Sua5.1 (Müller et al., 1999) maintained in Schneider's *Drosophila* medium supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. Approximately 5 x 10^5 cells per well were plated into 24-well plates 1 day prior to transfection and allowed to reach 60-70% confluency.

Qiagen effectene transfection reagent was used for transfection of constructs and the Dual-Luciferase Reporter Assay (Promega) used for promoter activity measurements. 600ng reporter constructs (*CYP9M10* upstream sequences in pGL3-Basic) and pGL3 without insert or 600ng shuttle construct pSLfa1180fa as controls (http://flybase.org/reports/FBmc0002761.html) were co-transfected with 1ng actin-*Renilla* internal control in 60µl DNA condensation buffer, 4.8µl enhancer and 6µl effectene in triplicate. Following incubation at 27°C for 48hrs and washing of cells with PBS, cells were harvested in 100µl passive lysis buffer (Promega) and luciferase activity measured on a luminometer (EG&G Berthold). Construct luciferase activity was normalised to *Renilla* luciferase activity.

2.6 Association of kdr *or the* CuRE1 *insertion upstream of* CYP9M10 *with permethrin resistance in Ghana*

Culex larvae were collected in May to early June 2011 from four sites in the Greater Accra region of southern Ghana (Ashaiman, Kpone, Labadi and Madina) and one site 80km distant (Koforidua) (GPS co-ordinates are provided in the supplementary material). Larval permethrin bioassays were performed on third to fourth instar larvae using the approach detailed above with the exception that assays were conducted in 125ml volumes and at permethrin concentrations ranging from 0.000015 to 3ppm. One hundred larvae were tested at each concentration point. Given that these were wild caught *Culex* from mixed species collections it was necessary to identify the larvae using a combination of morphological keys (Hopkins, 1952) and molecular assays (Smith and Fonseca, 2004). In addition an 830bp fragment of the 3' region of the mitochondrial cytochrome oxidase one (*COI*) gene was also amplified using the primer pair C1-J-2183 and TL2-N-3014 (Simon et al., 1994) and phylogenetic trees constructed to ensure that specimens clustered with known *Cx. quinquefasciatus*. Only confirmed *Cx. quinquefasciatus* were included in subsequent analyses. To perform a allele:phenotype association test a sample site-specific definition of resistant and susceptible was made (Table 1). Susceptible larvae were those killed at the lowest concentrations and resistant larvae those that survived exposure to the highest concentrations. These individuals were then screened for the genotype at codon 1014 of the VGSC using the pyrosequencing assay of Wondji et al. (2008) and for the presence of the *CuRE1* insertion upstream of *CYP9M10* using the protocol of Itokawa et al. (2010). Since the duplication of *CYP9M10* could be a cryptic genotypic variable a subset of samples were screened with the duplication diagnostic PCR of Itokawa et al. (2010). Association between the resistance phenotype and genotype was analysed using a chi-squared test in PopTools (Hood, 2008).

2.7 Screening for the CuRE1 *insertion in field populations of* Culex quinquefasciatus *from Africa*

Screens for the presence of the *CuRE1* element upstream of *CYP9M10* were conducted on additional populations using the assay of Itokawa et al. (2010). Confirmed *Cx. quinquefasciatus* from Nkhotakota Malawi (*N* = 12), Salima, Malawi (*N* = 7), Quelimane, Mozambique (*N* = 6), Lusaka, Zambia (*N* = 1) and Tororo, Uganda (*N* = 3 – n.b. these are siblings). A subset of samples were also amplified with primer pair 9M10UP3 and 9M10a, cloned into PJET1.2 (Fermentas) and sequenced.

3. Results

3.1 Bioassays

The LC_{50} for permethrin in the CqSF strain was 0.019 ppm (95% C.I.s 0.012-0.022) giving a resistance ratio (RR) for ISOP450 (LC_{50} 2.1ppm; Hardstone et al. 2009) relative to the susceptible CqSF strain of 112.

3.2 Gene expression and sequencing of CYP9M10 *and* CYP4H34

Differential expression of *CYP9M10* in the ISOP450 strain was confirmed by qPCR. Standard curves for *CYP9M10*, *CYP4H34* and *S7* exhibited efficiencies of 91.05, 99.75 and 106.75% respectively. Relative fold-changes of *CYP9M10* and *CYP4H34* ($\Delta\Delta C_t$ method corrected for efficiency) in the ISOP450 strain relative to CqSF were (Mean (95% CL)) 22.94 (16.67-29.21) and 0.6 (0.31-0.88) respectively. Thus, the ISOP450 strain has >20 fold expression of *CYP9M10* relative to the CqSF strain but marginally lower (0.6x) *CYP4H34* expression.

Cloning and sequencing of *CYP9M10* and *CYP4H34* was undertaken to investigate the role of allelic variation in the resistance phenotype. Cloning of *CYP9M10* revealed that the ISOP450 strain and CqSF strain differ in amino acid sequence from the Johannesburg strain genome reference strain (Vectorbase gene ID CPIJ014218) by four and seven amino acids respectively. The ISOP450 sequence differs from the CqSF strain at 10 amino acids (R143G, M156T, L232V, I245F, V279M, M288I, E289D, K356M, S422T, D445N). Critically, *CYP9M10* from both ISOP450 and CqSF encode full-length transcripts as does the Johannesburg reference strain (Itokawa et al. 2011). Therefore relatively greater susceptibility cannot be ascribed simply to a non-functional version of *CYP9M10* in CqSF, unlike the Ogasawara susceptible strain studied previously (Itokawa et al., 2010), which contains a deletion resulting in a premature stop codon.

CYP4H34 from ISOP450 strain differs from that of the Johannesburg strain (CPIJ011127) at 6 amino acids, CqSF CYP4H34 differs from CPIJ011127 at 3 amino acids and ISOP450 differs from the CqSF strain at 9 amino acids (T118S, K211M, T257A, V342L, V347D, T357I, V414A, E418K, K480N).

Alignments of CYP9M10 and CYP4H34 are given in Supplementary Figs. 1 and 2; Genbank accession numbers are JQ001925-JQ001928 for CqSF *CYP9M10*, ISOP450 *CYP9M10*, ISOP450 *CYP4H34* and CqSF *CYP4H34* respectively.

3.3 Recombinant expression and functional validation of CYP9M10 and CYP4H34

Through co-expression of both ISOP450 and CqSF CYP9M10 or CYP4H34 with AgCPR we isolated *E.coli* membranes with 0.08-0.51nmol P450/mg protein and CPR activity of 40-150 nmol cytochrome c reduced/min/mg protein. The majority of the P450 was functional as adjudged by the prominence of the P450 peak relative to the P420 peak in CO-difference spectra (see Supplementary Fig. 3). CYP9M10 and CYP4H34 membranes from both ISOP450 and CqSF clearly metabolized permethrin in an NADP+ dependent fashion (see Fig. 1 and Supplementary Fig. 4a-d). Three metabolite peaks (eluting at ≈7.62min, 8.88min and 10.2min) were evident following 60 min reactions in +NADPH reactions for both allelic variants of CYP9M10 (Fig. S4a-b), indicating that both are able to metabolise permethrin and that allelic variation does not contribute to resistance in the ISOP450 strain. In time-course experiments, these CYP9M10 metabolites increased in a NADPH- and time-dependant manner (Fig. 1A and 1B respectively). Weak metabolism of deltamethrin by CYP9M10 was noted, with minor metabolite peaks at 5.3min, 5.98min and 6.62min seen following 60 min reactions in +NADPH reactions only (Supplementary Fig. 4e-f).

Metabolite peaks for CYP4H34 eluted at 5.54min, 5.91min, 7.63min and 8.76min (Supplementary Fig. 4c-d) indicating that metabolism may involve a different reaction course for this enzyme. Both ISOP450 and CqSF CYP4H34 alleles were able to metabolise permethrin but there was no evidence for metabolism of deltamethrin by CYP4H34 expressed from either allele (Supplementary Fig. 4g-h).

3.4 Luciferase assay of region upstream of CYP9M10

The region upstream of *CYP9M10* from both the ISOP450 and CqSF was cloned and sequenced. The upstream region of ISOP450 amplified with primers 9M10UP5 and 9M10UP8 is 1502bp in length whilst that amplified from CqSF is 834bp. A number of SNPs and indels differentiate the upstream region of ISOP450 from CqSF (Supplementary Fig. 5) but the main difference between these regions is the 672bp sequence incorporating a fragment of a MITE (*CuRE1*) in the region upstream of ISOP450 *CYP9M10* that is absent from the region upstream of CqSF *CYP9M10*. In order to localise regulatory motifs, two different sized fragments immediately upstream of the *CYP9M10* start codon were amplified and cloned into the reporter gene vector pGL3. When used in reporter gene assays, inserts from both strains drove reporter gene expression, but expression driven by inserts derived from ISOP450 was 10x greater than that from CqSF (Fig. 2). Using fusion PCR we removed the *CuRE1* element whilst retaining all additional variation. Sequences lacking *CuRE1* drove expression at a similar level to those with the TE (Fig. 2) indicating that regulatory motifs are not located in this sequence.

3.5 Association of the MITE insertion upstream of CYP9M10 *with permethrin resistance in Ghana*

A total of 264 definitively confirmed *Cx. quinquefasciatus* larvae obtained from larval breeding sites across Greater Accra were assayed for permethrin susceptibility. LC_{50} based population-specific resistance ratios relative to the susceptible S-Lab strain are shown in Table 1 and ranged from 38-1,443. Due to differences in the population specific LC_{50} values, diagnostic concentrations varied for each population but were always chosen to establish a clearly distinct resistant and susceptible phenotype, whilst giving appropriate sample sizes for an association test ($N \approx 50$ per site).

There was a significant association between the *CuRE1* insertion upstream of *CYP9M10* and a permethrin resistant phenotype across all five sample sites combined ($p < 0.0001$; Table 1) and for

four of the five collection sites when analysed individually. Where significant, population specific odds ratios (ORs) for the *CuRE1* insertion ranged from 2.90 (Labadi) to 6.15 (Koforidua).

The duplication of *CYP9M10* described previously in the JPAL strain (Itokawa et al., 2011; Itokawa et al., 2010) could be a cryptic variable in studies of resistance association. To investigate this, a subset of Ghanaian samples (*N* = 24) was analysed for the presence of the duplicated *CYP9M10* (*CYP9M10v2*) using the Gen2F PCR of Itokawa et al. (2010). All positive samples exhibited the standard *CYP9M10*. Two samples were positive for *CYP9M10v2* indicating the duplicated copy is segregating in Ghana at low frequency.

Two SNPs in codon 1014 of the VGSC (TTA-TTT and TTA-TTC both L-F) were detected in these populations through pyrosequencing. There was no positive association of 1014F with resistance although a negative association was detected for the Madina population (Table 1).

3.6 Presence of CuRE1 *in additional African populations*

In screening of field-collected adult *Cx. quinquefasciatus* samples from Malawi, Mozambique, Uganda and Zambia we detected the presence of the inserted *CuRE1* allele in samples from Accra, Ghana, Lusaka, Zambia and Nkhotakota and Salima, Malawi, Quelimane, Mozambique and Tororo, Uganda. Following cloning and sequencing of representative alleles no variation was found in the 1107bp fragment containing the *CuRE1* allele from any location and all sequences were identical to that from ISOP450 (Supplementary Fig. 6). Thus, whilst the *CuRE1* element does not itself contain regulatory motifs, it can act as an easily scorable marker for the as yet unidentified regulatory regions in the ISOP450 upstream region. Non-*CuRE1* alleles exhibited variation, particularly in the vicinity of two poly-A tracts (Supplementary Fig. 6). Samples positive for the *CuRE1* insertion were also screened by PCR to determine the presence of *CYP9M10* or *CYP9M10v2* (duplicated *CYP9M10*) (Itokawa et al., 2010). Whilst the majority of samples were positive for *CYP9M10*, some were

positive for both *CYP9M10* and *CYP9M10v2* thus the duplication of *CYP9M10* is polymorphic in African populations.

4. Discussion

Using an *E.coli* system to express CYP9M10 and CYP4H34 from both the ISOP450 strain and the susceptible CqSF strain followed by *in vitro* metabolism, we have demonstrated the ability of both enzymes to metabolise permethrin. The evidence for *in vitro* metabolism and up-regulation presented here (see also Komagata et al., 2010), implicate *CYP9M10* as a causal gene underlying resistance in lab strains. CYP4H34 does not metabolise deltamethrin and CYP9M10 displays only very weak activity, correlating with the known phenotypic resistance profile of the ISOP450 strain (Hardstone et al., 2007). Differential metabolism of class I and class II pyrethroids has been seen for other CYP450s e.g. CYP6AA3 from *An. minimus* (Duangkaew et al., 2011). Improvements in our understanding of the structural mechanisms underpinning such differential activity may be gained from modelling of these P450s and we are currently pursuing this line of enquiry. Cytochrome P450s display high levels of nucleotide variability (Wilding et al., 2009) and allelic variants may underlie resistance. Both *CYP9M10* and *CYP4H34* exhibit alleles from the resistant and susceptible strains that differ at 10 and 9 amino acids respectively. However, we conclusively support the hypothesis of Hardstone et al. (2010) and Komagata et al. (2010) that resistance in the ISOP450 strain (and hence likely in the parental strain JPAL) is due to over-expression of *CYP9M10* and not allelic variants since both ISOP450 and CqSF derived alleles were capable of metabolising permethrin to the same extent. Interestingly, both CYP9M10 and CYP4H34 can metabolise permethrin although they appear to have differing actions, as evidenced by the differences in metabolite profiles. Whilst CYP4H34 is capable of metabolising permethrin, it does appear to have a much lesser role in the resistance phenotype. Although there is no difference in the metabolic activity of divergent alleles for both genes, unlike *CYP9M10*, *CYP4H34* is not over-expressed in the resistant (ISOP450) strain. Our estimate of >20-fold higher expression of *CYP9M10* in ISOP450 relative to CqSF (from qPCR) is similar in magnitude to the estimate of 11-fold over expression (*vs*. S-LAB) in a North American strain selected for permethrin resistance (Liu et al., 2011) but less than the 264-fold change found for JPAL *vs* Ogasawara (Komagata et al., 2010). It is however incompatible with the 1800-fold over-expression reported by

Hardstone et al. (2010) for ISOP450 *vs* S-LAB. Whilst *CYP9M10* can be induced by recent exposure (Liu et al., 2011) this cannot explain the levels estimated by Hardstone et al. (2010) which are much higher even than for tandemly duplicated genes e.g. esterase *E4* in aphids which exhibit fold changes of >60 (Puinean et al., 2010). Nevertheless, the over-expression encountered in our study is compatible with gene expression underlying the observed phenotype.

In the JPAL strain of *Culex* and the ISOP450 strain derived from JPAL, a partial miniature inverted transposable element (MITE), *CuRE1*, is inserted directly upstream of *CYP9M10* (Itokawa et al., 2010). The presence of this MITE correlates with survivorship in crosses of JPAL and a susceptible strain (Itokawa et al., 2010), but no direct evidence that this MITE contributes functional motifs has been generated previously. Using a luminescent reporter assay we have now shown that this upstream region from the resistant ISOP450 strain drives approximately 10-fold expression when compared to the region from CqSF which lacks the *CuRE1* element. When accounting for the *CYP9M10* duplication in the ISOP450 strain, this value is strikingly similar to the 20x fold-change we estimated by qPCR for the over-expression of *CYP9M10*. Whilst there are numerous SNPs/indels differentiating the upstream regions of ISOP450 and CqSF, the most notable difference is the presence of the 672bp region derived from the MITE suggesting that, as for the *Accord* element of *D. melanogaster* (Chung et al., 2007), this TE may contribute regulatory sequences. However, when the MITE is excised through fusion PCR, whilst retaining the SNPs and indels which differentiate the upstream regions of ISOP450 and CqSF, the 10-fold reporter expression is maintained, indicating that *CuRE1* does not provide regulatory elements. Whilst we cannot discount the possibility that *CuRE1* provides regulatory motifs active in other tissues (Sua5.1 are a haemocyte cell line and regulatory motifs may be tissue specific) it seems likely that the important regulatory motifs are present within the SNPs and indels which differentiate these sequences.

Genomic regions subjected to strong, recent selection pressure would be expected to show evidence of a selective sweep with reduced (or obliterated) diversity extending outward from the site of

selection to a distance dependent upon levels of recombination, strength of selection and time since emergence of the mutation e.g. (Catania et al., 2004; Karasov et al., 2010; Lynd et al., 2010; Saminadin-Peter et al., 2012). Through cloning and sequencing of the region upstream of *CYP9M10* in field populations we have shown that sequence identical to that found in the ISOP450 strain can be found in Ghana, Malawi, Zambia and Uganda. This suggests not only a ubiquitous pan-African distribution of the resistant allele but also a recent selective sweep (Lynd et al., 2010), which mirrors the situation for *Drosophila CYP6g1* where a selective sweep is indicated from worldwide sampling (Catania et al., 2004; Schlenke and Begun, 2004). A consequence of the selective sweep is that *CuRE1* is in perfect linkage disequilibrium with regulatory motifs present within the remainder of the upstream sequence. Thus, whilst *CuRE1* is not causal, it can act as a marker of the causative *cis*-regulatory elements. Itokawa et al. (2010) developed a simple diagnostic for the *CuRE1* insertion which has not been applied previously to wild populations. Through screening for the presence/absence of the TE insertion we have now demonstrated a strong association between presence of *CuRE1* and permethrin resistance in field-collected mosquitoes from 4 of 5 separate populations collected from Accra, Ghana. Since populations are separated by up to 80km, these are likely to represent at least partially independent populations. Effect sizes (odds ratios) are high – up to 6.15, comparable to the OR (≈ 6) for the known pyrethroid target site *L1014F kdr* mutation in a study of insecticide resistance in *An. gambiae* (Weetman et al., 2010). Whilst presence of *CuRE1* is associated with resistance, there is no association between resistance and genotype at codon 1014 of the voltage-gated sodium channel, and, indeed a negative association for the Madina population. It is possible that the phenotypic effect of these *kdr* mutations is relevant only in adult populations which were not the target of this study.

These data indicate that the assay of Itokawa et al. (2010) has relevance in natural populations. This therefore represents a marker for metabolic based resistance to insecticide that has proven applicability to wild collected populations. However, whilst this assay detects the presence of the

CuRE1 TE, it is not the TE that is causal. Hence, further work is necessary to dissect the *cis*-element in order to identify the causal regulatory motifs.

5. Conclusion

In this study, through heterologous expression of a cytochrome P450 from *Culex quinquefasciatus*, we implicate CYP9M10 as the enzyme responsible for metabolic resistance in the ISOP450 strain. Results of reporter assays demonstrate that whilst a transposable element is inserted upstream of *CYP9M10* in resistant mosquitoes, this does not provide regulatory motifs underlying expression suggesting that a re-evaluation of the assumption that TEs inserted upstream of CYP450s contribute functional motifs may be necessary. There is evidence of strong selection acting on the region upstream of CYP9M10 such that only a single resistant haplotype is found throughout Sub-Saharan Africa. Using a simple diagnostic to detect this haplotype we are able to demonstrate a strong association with resistance.

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Appendix. Supporting material

Supplementary material related to this article can be found online

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Figure Legends:

Figure 1. A) Metabolism of permethrin by CYP9M10 from ISOP450 with (P+) and without (P-) NADPH. *Cis*-permethrin elutes at ≈18min and *trans*-permethrin at ≈15min. B) Time series of *in vitro* metabolism of permethrin by ISOP450 CYP9M10 membranes. Peak area of peaks identified in 2A (7.8, 9.2 and 10.2minutes) are shown from +NADPH reactions. All peaks in –NADPH reactions did not increase with time but only the 7.8min peak is indicated for clarity.

Figure 2. Reporter gene activity (normalised to renilla fluorescence) in large fragments (amplified with 9M10UP5 & 9M10UP8) and small fragments (amplified with 9M10UP6 & 9M10UP8) from ISOP450 (+/- *CuRE1*) and CqSF, pGL3 and shuttle constructs (both controls). Error bars show +/- S.E. mean.

Population	LC_{50} in ppm (95% C.I.)	RR[a]	Phenotype	Phenotype definition	*CuRE1* alleles[b]			*kdr* alleles[c]		
					+CuRE1	*-CuRE1*	*P*	A	T or C	*P*
Ashaiman	0.0651 (0.041 - 0.105)	38	R	Survived exposure ≥ 0.15ppm	36	12	<0.0001	20	10	0.355
			S	Killed by exposure ≤ 0.015ppm	17	31		11	11	
Koforidua	0.3666 (0.215 - 0.625)	215	R	Survived exposure ≥ 1.5pm	45	9	<0.0001	12	14	0.969
			S	Killed by exposure ≤ 0.15ppm	26	32		10	10	
Kpone	0.95 64 (0.516 - 1.772)	563	R	Survived exposure ≥ 3ppm	46	8	0.484	24	24	0.985
			S	Killed by exposure ≤ 0.015ppm	40	10		19	21	
Labadi	2.4537 (1.788 - 3.367)	1443	R	Survived exposure ≥ 3ppm	48	12	0.0183	26	22	0.682
			S	Killed by exposure ≤ 1.5ppm	22	16		17	19	
Madina	0.2272 (0.184 - 0.281)	134	R	Survived exposure ≥ 3ppm	57	7	0.00038	15	41	$1.74x10^{-4}$
			S	Killed by exposure ≤ 0.15ppm	33	21		1	61	

Table 1. Association of *CuRE1* or *kdr* allele count with population specific permethrin resistance phenotype. [a] Resistance ratios relative to the S-Lab susceptible strain based upon S-LAB LC_{50} of 0.0017ppm (Hardstone et al., 2009). R = resistant. S = susceptible. [b] allelic counts of *CuRE1* positive (*+CuRE1*) and *CuRE1* negative samples (*-CuRE1*). [c] allelic counts for nucleotide at position 3 of codon 1014 of the voltage gated sodium channel (numbering according to the housefly *para* sequence, GenBank accession no. X96668). TT<u>A</u> = leucine TT<u>C</u>/TT<u>T</u> = phenylalanine.

Figure 1

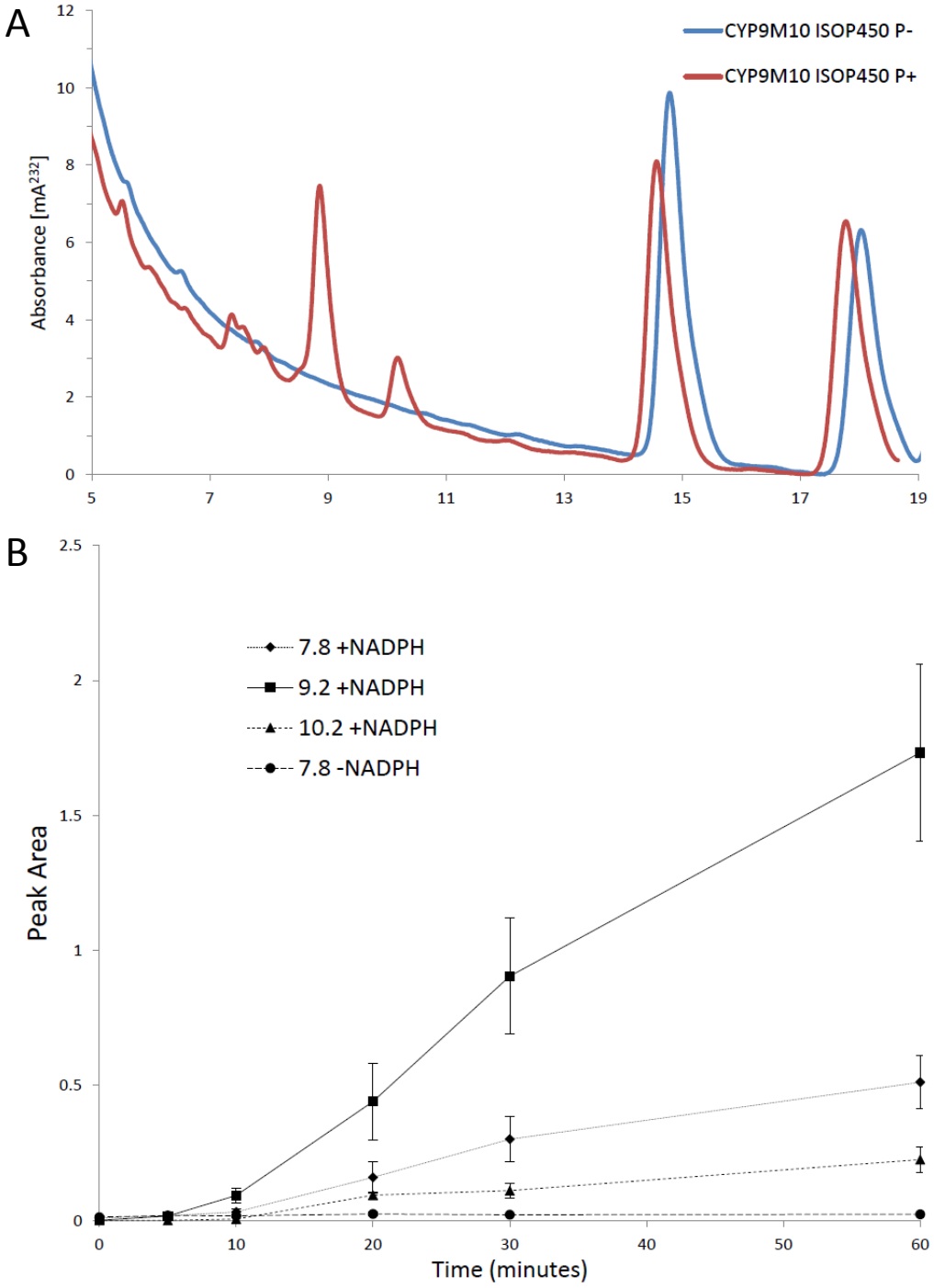
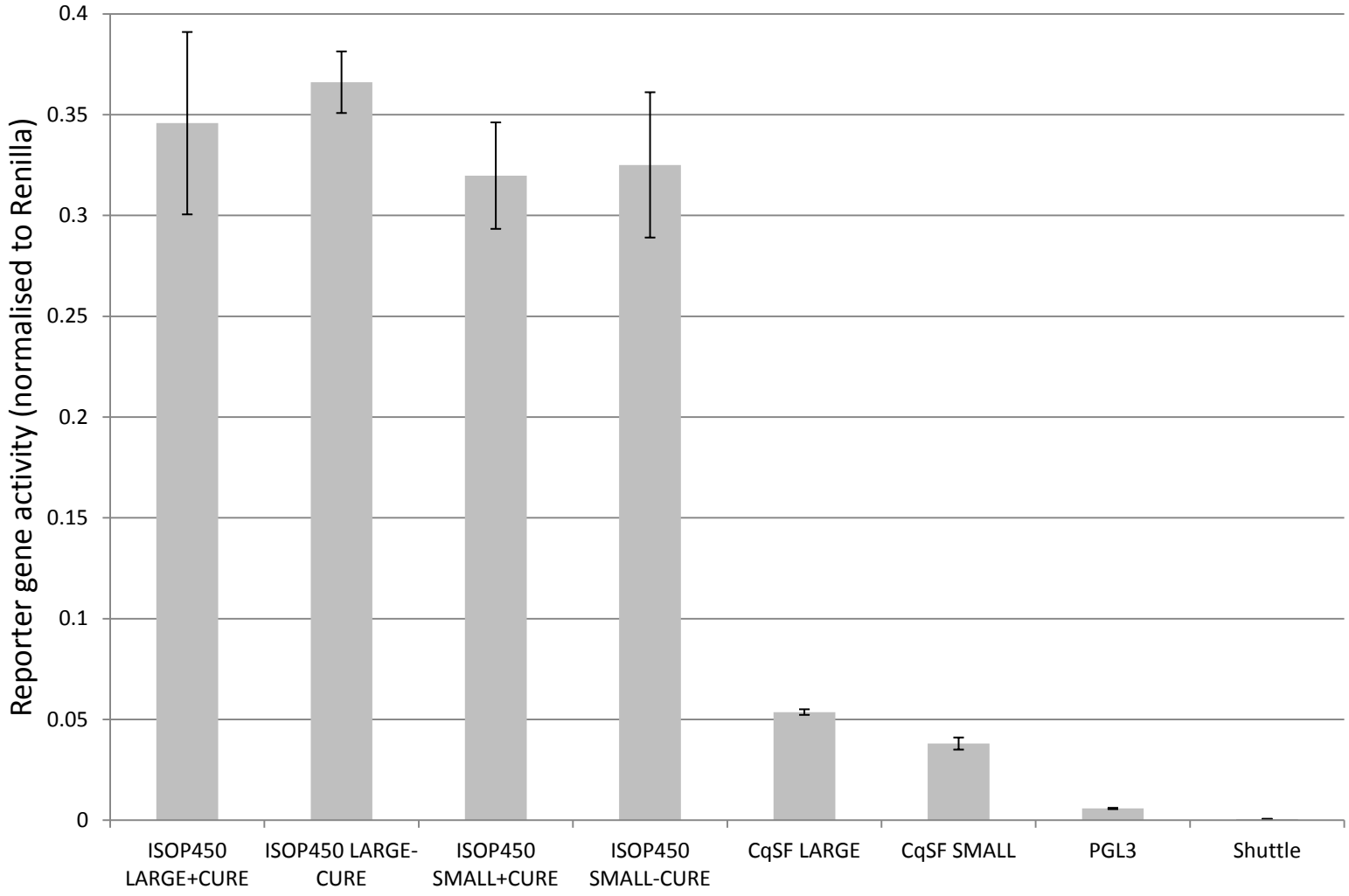

A
CYP9M10 ISOP450 P-
CYP9M10 ISOP450 P+
Absorbance [mA232]
12
10
8
6
4
2
0
5
7
9
11
13
15
17
19
B
7.8 +NADPH
9.2 +NADPH
10.2 +NADPH
7.8 -NADPH
Peak Area
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
Time (minutes)

Figure 2


Reporter gene activity (normalised to Renilla)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ISOP450 LARGE+CURE
ISOP450 LARGE-CURE
ISOP450 SMALL+CURE
ISOP450 SMALL-CURE
CqSF LARGE
CqSF SMALL
PGL3
Shuttle