Microarray analysis of a pyrethroid resistant African malaria vector, *Anopheles funestus,* from southern Africa

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Abstract

Anopheles funestus is one of the major malaria vectors in southern Africa and several populations in this region are resistant to pyrethroids. The current study uses a microarray based approach to identify genes up-regulated in the pyrethroid resistant population, FUMOZ, from Mozambique. As the full set of detoxification genes in *An. funestus* are unknown, this study investigated the utility of the *An. gambiae* 'detox chip' to screen for differentially expressed detoxification genes in *An. funestus*. Differential expression of detoxification genes in three day old adult females and males from the FUMOZ resistant strain and the FANG susceptible strain was identified using the *An. gambiae* 'detox chip'. After optimization of the hybridization conditions, over 90% of the probes showed a positive signal. Only three genes were significantly ($p<0.001$) differentially expressed in the females, *CYP6P9* (5.4-fold), *COI* (2.7-fold) and *CYP6M7* (1.8-fold). The same genes were also significantly differentially expressed in the adult males, *CYP6P9* (6.0-fold), *COI* (2.9-fold) and *CYP6M3* (3.6-fold) together with an additional 21 transcripts. Quantitative PCR (qPCR) analysis was conducted to validate the microarray results. This study demonstrated that heterologous hybridization is a helpful tool in identifying detoxification genes differentially expressed in *An. funestus* strains.

Keywords:

Microarrays*, An. funestus,* 'detox chip', pyrethroid resistance, cytochrome P450.

1. Introduction

In 2008 the World Health Organization [1] reported that an estimated 247 million people are affected worldwide with malaria. Most malaria deaths occur in Africa and this situation results from both the epidemiological situation in Africa and limited control activities during the past decades. The lack of adequate health services often results in deficiencies in treatment and this is compounded by increasing drug resistance [2]. Insecticide resistance in the mosquito vectors is increasingly impacting on malaria control interventions [3; 4]. Malaria control in southern Africa is largely based on the use of insecticide treated bed nets (ITNs) or indoor residual spraying (IRS) with pyrethroids being the insecticides of choice. There have been reports of insecticide resistance in malaria vectors in southern Africa and these include DDT and pyrethroid resistance in *Anopheles arabiensis* in South Africa [5; 6] and *An. funestus* resistant to pyrethroids and carbamates in South Africa and Mozambique [7; 8]. The development of pyrethroid resistance in *An. funestus* resulted in a malaria outbreak in South Africa between 1996 and 2000 [7].

Two major resistance mechanisms are largely responsible for insecticide resistance i.e. target-site resistance and metabolic resistance [9]. To date there have been no reports of target site resistance to pyrethroids in *An. funestus*. Metabolic resistance is characterized by increased activity of detoxifying enzymes such as esterases, cytochrome P450 monooxygenases and glutathione-S-transferases (GSTs) [9; 10]. All three enzyme groups are encoded by large gene families and identifying the individual genes associated with resistance is a very laborious exercise. This task was facilitated for *An. gambiae* by the

development of a custom microarray specifically designed to detect transcription variation in genes associated with insecticide resistance [11]. This array was named the 'detoxification chip' (detox chip'). The 'detox chip' has been utilized for screening *An. gambiae* populations from Kenya and Zanzibar [11], Benin [12], Nigeria [13] and Ghana [14; 15] and has also been successfully used for *An. arabiensis* from Cameroon [16] and *An. stephensi* from Dubai [17].

Biochemical assays and synergists have been used previously to characterize the resistance mechanisms in the FUMOZ-R pyrethroid resistant strain from Mozambique [8]. These studies implicated elevated cytochrome P450 activity as the main mechanism. This was later supported by qPCR [18] and genetic mapping studies [19] which both identified the P450 gene, *CYP6P9*, as being highly over-expressed in the resistant FUMOZ-R strain.

In this study we investigated the use of the *An. gambiae* 'detox chip' in determining potential genes associated with insecticide resistance in a southern African *An. funestus* resistant strain (FUMOZ-R) and a susceptible strain (FANG). Selected significantly over-expressed genes from the microarray results were validated using qPCR.

2. Materials and methods

2.1. Mosquito strains

Two *An. funestus* strains were used in this study. The FUMOZ-R strain originated from Mozambique and has been maintained under selection pressure with permethrin. FANG originates from Angola and is susceptible to all known insecticides. Details on the insecticide resistance status of these colonies can be found in the study by Hunt *et al.* [20]. Both strains are maintained in standard insectary conditions of 25°C with 80% relative humidity and 12 h day/night, 45 min dusk/dawn lighting cycle.

2.2. Sample preparation and microarray hybridizations

Female and male *An. funestus* adults were separated on day of emergence and fed on 10% sugar solution until they were three days old (without prior exposure to pyrethroids). The transcription levels in the three day old resistant FUMOZ-R adult females were compared to the susceptible females of the same age. The same was done for the males. Each comparison in all experiments consisted of three independent biological replicates and two technical repeats which included dye swaps to control for dye bias. There were four within-array replicate spots per microarray slide. Total RNA was extracted from three batches of fifteen three day old adult females or males using the PicoPure ^{TM} RNA Isolation kit (Arcturus) according to manufacturer's instructions. Amplified mRNA (amRNA) was synthesized using the RiboAmp^{TM} RNA Amplification Kit (Arcturus) according to manufacturer's instructions. amRNA was measured using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA) and the quality of the RNA was assessed on a 0.8% gel.

The RNA and primer mix consisting of 8µg of amRNA for each of the samples 2µl mRNA spike mix (Universal Lucidea Scorecard, Amersham), random hexamers (Invitrogen) and water was incubated for 5 minutes at 70ºC. The RNAs mix were reverse transcribed into Cy-labeled cDNAs using Cy3 or Cy5-dUTPs (Amersham), DTT, dT-NTPs, RNAsin and Superscript III (Invitrogen). The samples were incubated at 50ºC for 2 ½ hours. A mixture of 1M NaOH/ 20mM EDTA was added to the labeled cDNAs to stop the reaction. The Cy-labeled cDNAs that were to be hybridized together were pooled and purified using the Cyscribe™GFX™ Purification kit (Amersham). The concentration of the cDNAs and the binding efficiencies of the Cy-dyes were determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA). Samples that had a low cDNA yield (<15ng/µl) and poor dye incorporation (<0.1 pmol/µl of each dye) were discarded. 5µg of poly dA oligo (Amersham Biosciences) were added to the purified targets to reduce nonspecific hybridization. The labeled samples were dried in a speed vacuum centrifuge for 1 hour at 30ºC and re-suspended in 15.5µl of hybridization buffer (Corning). The targets were denatured by incubation at 95°C for 5 minutes. The *An. gambiae* 'detox chip' was pre-treated using the Universal Hybridization kit (Corning), according to manufacturer's instructions. However the wash solutions were made up using three times the amount of components as stated in the protocol by David *et al.* (2005). The target mix was added to the microarray chip and hybridizations were performed at 38°C for 16 hours.

After the incubation the slides were washed using the Universal Hybridization kit (Corning). The slides were pre-soaked in wash solution 1 for one to two minutes at 38°C,

followed by a second wash of solution 1 for 2 ½ minutes and finally incubated in wash solution 2 at ambient temperature for 5 minutes. The slides were subjected to three washes in fresh wash solution 3 at ambient temperature for one minute. Excess wash solution was removed from the slides by centrifugation at 2500 rpm for two minutes.

2.3. Microarray scanning and data analysis

The slides were scanned using the Genepix 4000B scanner (Molecular Devices, USA), where the PMT values were adjusted to give a ratio of 1/1. The quality of spots and background intensities were examined and corrected using the Genepix Pro 6.0 software (Axon instruments, USA).

The raw intensity values were analyzed using the limma software package version 2.12.0, from the BioConductor project, in the R programming environment [21]. Image plots were generated to inspect the variation of background values across the arrays. Background correction was carried out using the 'normexp' method [22] with an offset of 50. Within array normalization was performed, using the 'control' method in limma to fit a global loess curve through a set of non-differentially expressed control spots. This curve was applied to all the other spots, thereby normalizing the M-values for each array separately [23]. Between array normalization was performed using the 'Aquantile' method to ensure that the A values have the same empirical distribution across arrays. To visualize the effect of normalization, MA-plots were used. The limma package uses empirical Bayes analysis to assess differential expression, by calculating a *B*-statistic, a moderated *t*-statistic and an adjusted *p*-value for each gene [24]. A top table was

generated which revealed the highly differentially expressed genes for both the strains of interest. Genes exhibiting adjusted *p*-values <0.001 and fold-changes ≥1.5 were considered as statistically significant over-expressed genes. The data was deposited into the vectorbase database (https://www.vectorbase.org).

2.4. Quantitative Real Time PCR (qPCR)

Quantitative PCR analysis was carried out on three day old FUMOZ-R and FANG females and males to verify the results obtained from the microarray experiments. qPCR was only carried out on the three up-regulated genes in the females, and the same was done for the males. The remaining up-regulated genes in the males will be carried out in future studies. Primers were designed for genes *CYP6M7* and *COI*. The primer pair used for the *CYP6P9* gene was a gene-specific primer set [25] subsequent to the discovery of a duplicate gene, *CYP6P13* [26]. The cDNA probe sequence was aligned to both the *CYP6P13* (GenBank accession number: EF152577) and *CYP6P9* (GenBank accession number: EU450763) gene. The cDNA probe on the microarray slide had a 81% sequence identity to both *CYP6P9* and *CYP6P13*. Due to sequences similarities between *CYP6P9* and *CYP6P13*, gene-specific primers were used for both *CYP6P13* and *CYP6P9*.

2.4.1. RNA Isolation

The RNA samples obtained from the extractions used in the microarray experiments were used for the qPCR part of the study. The samples were quantified using a Nanodrop ® Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

2.4.2. cDNA synthesis

Total RNA was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, 4387406). The quality and quantity of cDNA was measured using the Nanodrop ® Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and the cDNA samples were stored at -70ºC until further use.

2.4.3. Primer Design

Primer sequences for *CYP6P9* and CYP6P13 were obtained from the Christian *et al.* [25] study. Primers were designed for the *CYP6M7* (Accession number: AY729660.1) and COI (Accession number: AY423059.1) genes using the *An. funestus* gene sequences. These primers were designed using Beacon Designer 3.0 software (Biorad, Hercules, CA, USA). The *rsp 7* (EF450776) primers was used as a reference gene to normalize data. Two different sets of primers were designed for the *CYP6M7* gene (Table 1). However amplification of the product was unsuccessful after numerous attempts to optimize the cycling conditions, primer and cDNA concentrations.

2.4.4. Quantification of CYP6P9, CYP6P13, CYP6M7 and COI

The qPCR experiments were performed using the Bio-Rad CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A total volume of 25µl containing 12.5µl IQ™ SYBR supermix (Bio-Rad, 1708882), 4µl primer (2.5µM), 1µl cDNA (50ng) was used per reaction. A 2-fold serial dilution of cDNA was used for the standard curve reaction. Serial dilutions of cDNA for the standard curve were prepared.

Table 1 Primers used in Quantitative PCR (qPCR).

Gene	**Primer Sequence**	**Fragment Length (bp)**	**qPCR Annealing/Detection Temp (°C)**	**Reference**
COI	**Fwd** 5' ATG GAG CAG GAA CAG GAT GAA CAG 3' **Rev** 5' AAT CAA CTG AAG CAC CAG CAT GAG 3'	75	59.40/79.00	This paper
CYP6M7	**Fwd1** 5'GAA GTG CTG GAA CGT CAT AAC 3' **Rev1** 5' CGG ATA TTT ACG CAG GCT TTC 3' **Fwd2** 5' TCA GAT TCC GAA AGA AAG C 3' **Rev2** 5' ATC ACG ATG AAT CGC ATA C 3'	* *	* *	This paper
CYP6P9	**Fwd** 5' AGA TGT GAT TGG CAC CTG T C 3' **Rev** 5' TCG ATA TTC CAC CGT TTC CT 3'	232	55/ 82.00	Christian *et al.,* 2010
CYP6P13	**Fwd** 5' CTG GAT CTC CTA ATT ATG ATG AAG TTT TTC 3' **Rev** 5' GTT CAC CGT CTC GCG GAC T 3'	132	59.1/ 81.00	Christian *et al.,* 2010
rsp 7	**Fwd** 5' TTA CTG CTG TGT ACG ATG CC 3' **Rev** 5' GAT GGT GGT CTG CTG GTT C 3'	135	**/ 85.50	Amenya *et al.,* 2008

* No amplification.

** Annealing temperature for the rsp 7 gene is equivalent to the respective genes.

The cycling conditions for each primer set are presented in Table 1. Each biological sample was repeated three times on a plate. Three biological repeats were done on different days. The data was analyzed using the relative quantification method [27].

3. Results

A previous study [25] has shown that both females and males from the resistant strain, FUMOZ-R, are resistant to permethrin, although mortality rates varied between the two sexes. Therefore gene expression was determined in both three day old adult females and males of the *An. funestus* FUMOZ-R (resistant) and FANG (susceptible) strains using the *An. gambiae* 'detox chip'. Minor adjustments to the original protocol were made in order to maximize hybridization of the targets to the chip. The hybridization temperature was reduced from 42ºC to 38ºC and lower stringency wash buffers and shorter washing times were used. These optimized conditions were similar to those used for cross-species hybridization to *An. stephensi* [17]. After optimization of the hybridization conditions over 90% of the spots on the slide were detected as opposed to 55% obtained when using the more stringent original hybridization conditions described by David *et al.* [11]. The percentage of probes detected was obtained by manually counting the number of fluorescent spots on the slides after hybridization.

Genes were considered to be differentially expressed if they demonstrated a ≥1.5-fold change in expression between the two strains and a *p*-value of <0.001 over the entire experiment. Out of a total of 254 gene probes on the *An. gambiae* 'detox chip', 1.18% of genes were significantly differentially expressed in the females as opposed to 9.45% in

Table 2 List of genes differentially expressed in females and males of the resistant *An. funestus* strain, FUMOZ-R. Gene names provided in table are as per *An. gambiae* naming, except for those where the *An. funestus* specific name has been identified (Amenya *et al.* 2005) which appears in brackets behind *An. gambiae* names.

Gene Name	**Group**	**Vectorbase Accession Number**	**Fold Change**	**Adj. *P-value***
Females				
CYP6P3 (CYP6P9)	Cytochrome P450	AGAP002865	5.4	2.84×10^{-07}
COI	Cytochrome oxidase I	DQ465331 (GB)	2.7	5.11×10^{-06}
CYP6M3 (CYP6M7)	Cytochrome P450	AGAP008213	1.8	8.82×10^{-04}
Males				
CYP6P3 (CYP6P9)	Cytochrome P450	AGAP002865	6.0	3.99×10^{-14}
CYP6M3 (CYP6M7)	Cytochrome P450	AGAP008213	3.6	4.13×10^{-08}
CYP6M2 (CYP6M8)	Cytochrome P450	AGAP008212	3.0	8.46×10^{-09}
COI	Cytochrome oxidase I	DQ465331 (GB)	2.9	5.21×10^{-12}
SOD2	Superoxide dismutase	AGAP005234	2.5	2.7×10^{-06}
GSTS1-1	Glutathione S-transferase	AGAP010404	2.5	5.21×10^{-12}
TRX1	Thioredoxin	AGAP009584	2.4	6.74×10^{-04}
Actincytopl	Actin	TIGR: TC48694	2.3	5.96×10^{-04}
CYP6R1	Cytochrome P450	AGAP008205	2.2	1.30×10^{-06}
TRX3	Thioredoxin	AGAP003338	2.2	6.74×10^{-04}
Cytochrome_C	Cytochrome C	AGAP009537	2.2	1.06×10^{-07}
CYP12f2	Cytochrome P450	AGAP008021	2.2	2.92×10^{-04}
CYP6AG1	Cytochrome P450	AY745223 (GB)	2.0	1.36×10^{-04}
CYP9J5 (CYP9J11)	Cytochrome P450	AGAP012296	2.0	6.54×10^{-06}
MnSOD1	Superoxide dismutase	AGAP010517	2.0	2.30×10^{-05}
SP8898	Serine protease	AGAP003642	1.9	2.73×10^{-04}
CYP9M1	Cytochrome P450	AGAP009374	1.8	4.73×10^{-05}
GSTS1-2	Glutathione S-transferase	AF513639 (GB)	1.7	6.74×10^{-04}
CYP6Z1	Cytochrome P450	AGAP008219	1.7	1.30×10^{-06}
CYP6AG2	Cytochrome P450	AY745224 (GB)	1.6	8.50×10^{-04}
GSTMS3	Glutathione S-transferase	AGAP009946	1.6	1.01×10^{-03}
CYP6M1 (CYP6M1)	Cytochrome P450	AGAP008209	1.6	5.96×10^{-04}
GSTD2	Glutathione S-transferase	AGAP004165	1.6	3.18×10^{-05}
CYP9J3 (CYP9J12)	Cytochrome P450	AGAP012291	1.5	7.71×10^{-04}

males. No genes were found to be over-expressed in both females and males from the susceptible *An. funestus,* FANG, strain. In the resistant *An. funestus* FUMOZ-R stain, however, three genes were differentially expressed in the females (Figure 1). The gene with the highest fold change in expression in the females was a cytochrome P450 gene *CYP6P9* (*CYP6P3* orthologous gene in *An. gambiae*) (5.4-fold) (Table 2). The cytochrome oxidase I gene, *COI*, was 2.7-fold differentially expressed and the third gene also a member of the cytochrome P450 enzyme family, *CYP6M7* (*CYP6M3* orthologous gene in *An. gambiae*) was 1.8-fold differentially expressed.

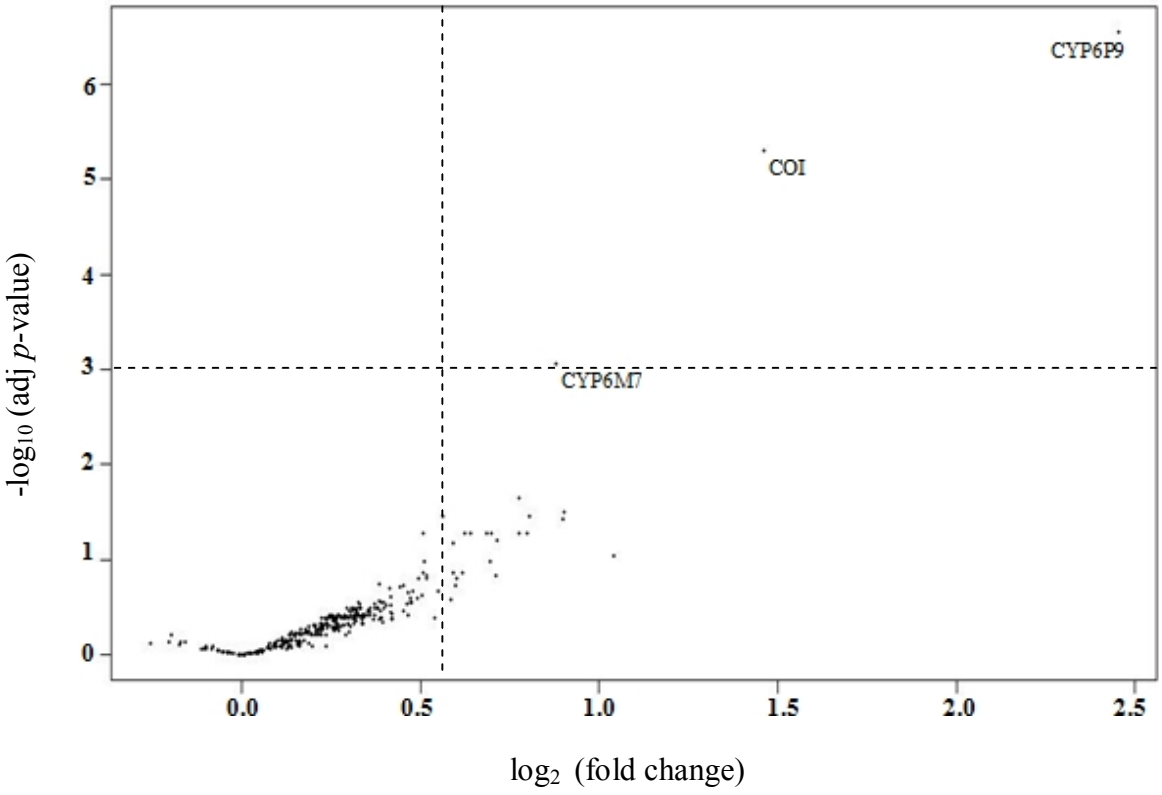

Figure 1 Volcano plot representing genes differentially expressed in females of the *An. funestus* strain, FUMOZ-R.
The horizontal line represents the cut off for the level of significance α=0.001 and vertical lines indicate cut off for the 1.5-fold change threshold.

A total of 24 genes were significantly differentially expressed in males from the resistant FUMOZ-R strain (Table 2). Fifty percent of these genes (n=12) belonged to the cytochrome P450 enzyme group, 17% (n=4) to the glutathione-S-transferase, 8% (n=2) to the Copper-Zinc (Cu-Zn) superoxide dismutase, 8% (n=2) to the thioredoxin group and the remaining 17% (n=4) to other groups. Three genes that were differentially expressed and common to both sexes include the cytochrome P450 genes, *CYP6P9* (6.0-fold), *CYP6M7* (3.6-fold) and cytochrome oxidase I, *COI* (2.9-fold) (Figure 2). Other differentially expressed genes in the males are shown in Table 2.

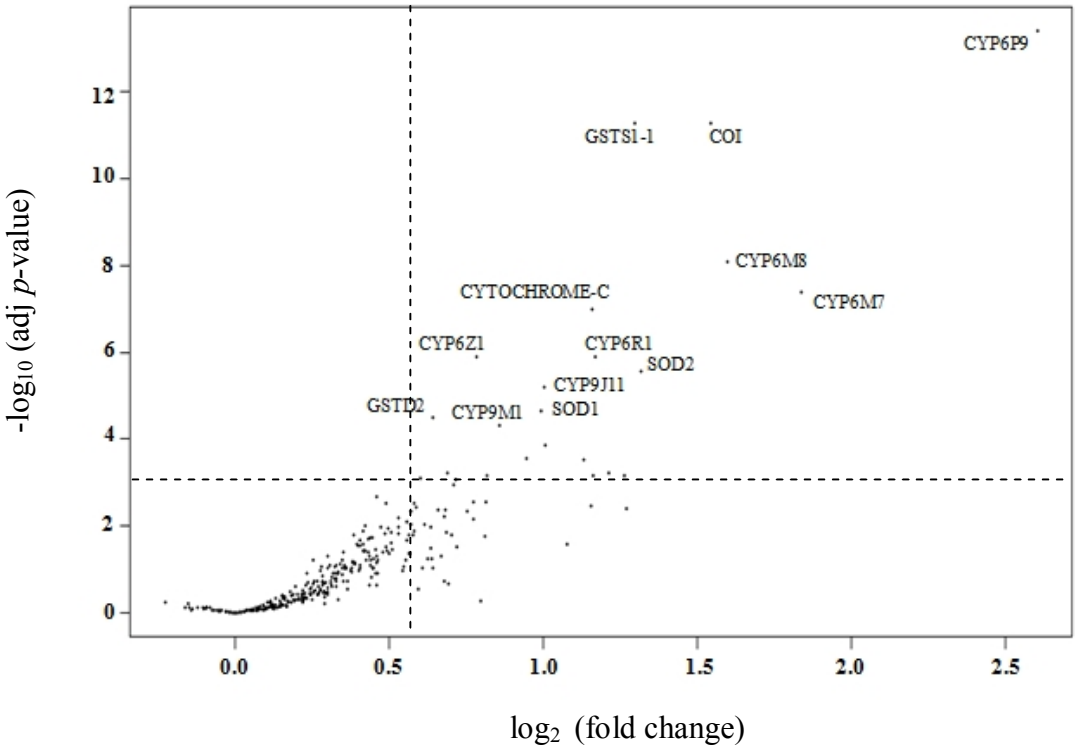

Figure 2 Volcano plot representing genes differentially expressed in males of the *An. funestus* strain, FUMOZ-R.
The horizontal line represents the cut off for the level of significance α=0.001 and vertical lines indicate cut off for the 1.5-fold change threshold.

Out of a total of 254 genes on the *An. gambiae* 'detox chip', 18 probes failed to produce any signal on the array (Table 3). Five of these genes, *CYP306A1*, *CYP6P4*, *CYP4C28*, *CYP305A3*, and *CYP6Z2* belonged to the cytochrome P450 group. Only two genes belonged to the GST group, *GSTD4* and *GSTD3* and the peroxidase (HPX) group, *HPX7* and *HPX5A*. However a large number of genes that did not bind to the microarray slide belonged to the carboxylesterase group. These include *COEAE1G*, *COEJHE1E*, *COEunkn*, *COEAE1F*, *COEAE7G*, *COEBE2O* and *COEBE4C*. The remaining genes were superoxidase dismutase (*SOD3B*) and the serine protease gene (*SP21408*).

Some sequence data for detoxification genes is available for *An. funestus* [18] enabling the degree of identity between the *An. gambiae* probes and their putative *An. funestus* orthologs to be determined. The probe sequences of the genes on the *An. gambiae* 'detox chip' were aligned against the known *An. funestus* sequences. The percentage identities were calculated for genes *COI, CYP6M1, CYP6M8, CYP6P4, CYP9J11, CYP9J12, CYP6P9* and *CYP6M7* (Table 4). All genes had a percentage similarity of >40%. The genes with the highest percentage similarity were genes *CYP6P9* (81%), *CYP6P13* (81%), *CYP6M1* (79.4%) and *CYP6P4* (75.0%). Surprisingly no signal was detected for *CYP6P4* despite the 75% sequence similarity between the two species.

The low level of sequence identity between some of the probes and their putative target genes in *An. funestus* suggests that some care should be taken in the interpretation of the results, particularly given the high level of sequence identity between some detoxification genes in the same species [18]. The possibility that the microarray results are confounded

Table 3 List of genes that did not hybridize onto the *An. gambiae* 'detox chip' in both the females and males of the resistant and susceptible *An. funestus* strain, FUMOZ-R and FANG.

Gene Name	**Group**	**Vectorbase accession number**
CYP306A1	Cytochrome P450	AGAP004665
CYP6P4	Cytochrome P450	AGAP002867
CYP4C28	Cytochrome P450	AGAP010414
CYP305A3	Cytochrome P450	AGAP005657
CYP6Z2	Cytochrome P450	AGAP008218
SOD3B	Superoxide dismutase	AGAP010347
GSTD4	Glutathione S-transferase	AGAP004381
GSTD3	Glutathione S-transferase	AGAP004382
COEAE1G	Carboxylesterases	AGAP006700
COEJHE1E	Carboxylesterases	AGAP005833
COEunkn	Carboxylesterases	AGAP011509
COEAE1F	Carboxylesterases	AGAP006227
COEAE7G	Carboxylesterases	AGAP006728
COEBE2O	Carboxylesterases	AGAP001101
COEBE4C	Carboxylesterases	AGAP005370
HPX7	Peroxidase	AGAP004036
HPX5A	Peroxidase	AGAP000051
SP21408	Serine protease	AGAP004015

by cross hybridization of probes to multiple genes can not be excluded. Therefore qPCR was performed to validate the results from the genes found over-expressed in both sexes.

Table 4 Percentage similarity between cDNA probes on *An. gambiae* 'detox chip' and the *An. funestus* genes

***An. funestus* gene name**	***An. gambiae* gene name**	**Percentage DNA similarity (%)**	***An. funestus* gene accession number**
COI	*COI*	50.3	AY423059.1
CYP6M1	*CYP6M1*	79.4	AY987356.1
CYP6M8	*CYP6M2*	49.4	AY729660.1
**CYP6P4*	*CYP6P4*	75.0	EU852651.1
CYP9J11	*CYP9J5*	52.3	AY729662.1
CYP9J12	*CYP9J3*	43.5	AY729663.1
CYP6P9	*CYP6P3*	81.0	EU450763
CYP6P13	*CYP6P3*	81.0	EF152577
CYP6M7	*CYP6M3*	54.1	AY729660.1

* Indicates gene that did not produce any signal on the array

3.1. Validation

Quantitative PCR (qPCR) was used to validate the microarray results for the three genes over-expressed in both sexes. The up-regulation of *CYP6P9* and *COI* in the resistant FUMOZ-R strain in both males and females is presented in Figure 3A and Figure 3B respectively. The resistant males showed a fold change of 51 for the *CYP6P9* gene, 15 for *CYP6P13* and one for *COI* when qPCR analysis was performed. The females also showed the highest fold change in *CYP6P9* (67), followed by *CYP6P13* (8) and 4 for the *COI* gene.

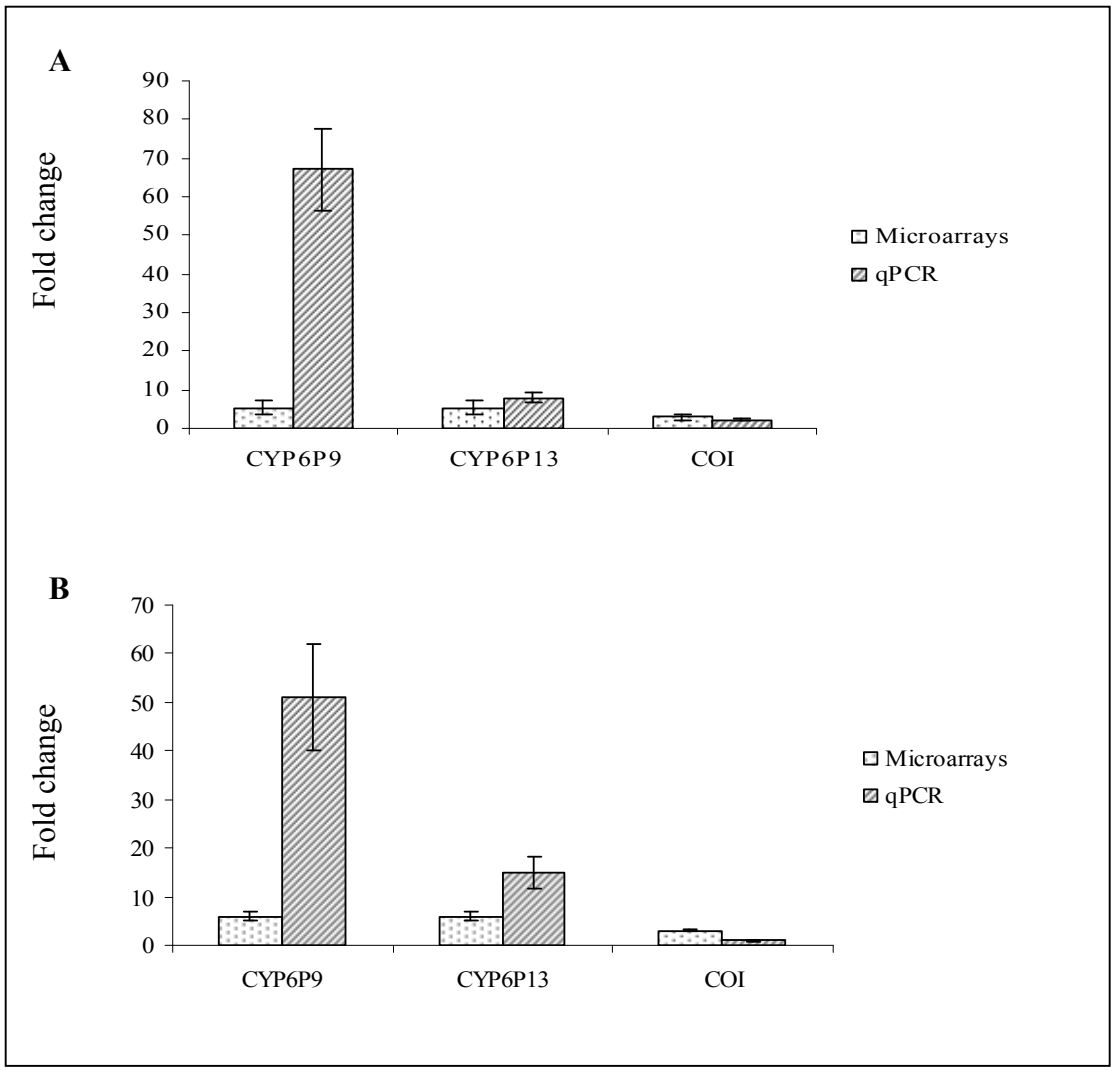

Figure 3 Comparison of qPCR and microarray results for *CYP6P9, CYP6P13* and *COI* genes in females (A) and males (B).

Comparing the fold over-expression between the qPCR and microarray analysis of the three genes within each sex shows that only *CYP6P13* and *COI* (females) were statistically similar (Figure 3). However each gene is over-expressed in the resistant strain in both males and females although at different levels. One of the reasons for this could be due to the effects of the low stringency hybridization conditions for microarray analysis and this could affect fold over-expression values. The sequence of the probes

present on the array for each gene is different to the sequence of the primers designed for the *CYP6P9* and *COI* genes for the *An. funestus* samples. This could be another contributing factor to the differences seen.

Two sets of primers were designed for the *CYP6M7* gene based on sequence information from the partial sequence of this *An. funestus* gene [18] (Table 1). However amplification of the product was unsuccessful for both primers even after numerous attempts.

4. Discussion

Gene expression in three day old *An. funestus* females and males was determined using the *An. gambiae* 'detox chip' developed by David *et al.* [11]. This study used cross-species (heterologous) hybridization of the *An. funestus* samples to an *An. gambiae* microarray platform due to the fact that unlike *An. gambiae*, a fully sequenced genome for *An. funestus* is currently unavailable. Many studies have successfully evaluated gene expression using non species-specific arrays due to lack of availability of arrays. Moody *et al.* [28] used human microarrays to study the gene expression in pigs. They found that the reproducibility of microarray hybridization of pig cDNA to human microarrays was high. Cross-species hybridization has also been conducted on rhesus macaque monkeys using a human high-density Affymetrix oligonucleotide array [29]. These results confirmed that a large number of genes were differentially expressed, thereby validating the use of cross-species hybridizations for nonhuman primate studies. Adjaye *et al.* [30] used human and bovine fetal brain target samples on a human cDNA microarray. Differentially expressed genes were obtained and the study proved that using tissues from

across species to identify co-expressed orthologous genes on a human microarray platform is possible.

The *An. gambiae* 'detox chip' has been successfully used to determine the transcriptional analysis of pyrethroid resistance in *An. stephensi* [17] and *An. arabiensis* [16]. This is the first study to employ microarrays to determine the genetic basis of pyrethroid resistance in *An. funestus.* The heterologous hybridization approach was used successfully in this study and approximately 90% probes were detected. Three genes were differentially expressed in both sexes, *CYP6M7*, *CYP6P9* and *COI; COI, CYP6P9* and its duplicate gene *CYP6P13,* were confirmed to be over-expressed using qPCR analysis. Although statistically the gene expression for the *COI* gene is different between the two methods for the males, the gene was still differentially expressed in the resistant strain. Although the over-expression of *COI* is not significantly high, the role of this gene needs to be investigated.

The fact that *CYP6M7* could not be amplified from *An. funestus* mRNA could possibly imply that it is a pseudogene. However, the lower hybridization stringencies can result in hybridization of another closely related P450 gene to the *CYP6M7* probe. This hypothesis can only be validated when a full genome of *An. funestus* is available. Wondji *et al.* [19] also attempted to amplify the *CYP6M7* gene using a different primer set from the ones used in this study and also failed to obtain amplicons. The *CYP6M7* gene needs to be further investigated.

The *CYP6P9* gene has been implicated in playing a role in insecticide resistance in *An. funestus.* In 2008, Amenya *et al.* [31] found the *CYP6P9* gene to be over-expressed in the egg and adult stages of a pyrethroid resistant laboratory strain (FUMOZ-R) originating from southern Mozambique. Wondji *et al.* [32] identified a quantitative trait locus (QTL) associated with pyrethroid resistance in the same *An. funestus* strain. He found that these QTL markers contained a cluster of P450 genes including *CYP6P9* and that the chromosomal position of *CYP6P9* is associated with permethrin resistance. In 2009 the same author [19] reported that the *CYP6P9* gene was 25 times over-expressed in the FUMOZ-R strain in females and that it was tandemly duplicated and this was suggested to contribute to insecticide resistance. Interestingly the *CYP6P3* (*An. gambiae* ortholog) was also found to be differentially expressed (2.82-fold) in field caught *An. gambiae* adults from Ghana [15]. The *CYP6P3* gene was also found at significantly high levels (2.6-fold) in *An. arabiensis* adults from Cameroon [16].

The observation that additional genes were up-regulated in males than in females in the FUMOZ-R strain was surprising given that resistance is higher in females than males of this strain [25]. It is possible that these 21 additional differentially expressed genes are unrelated to the resistance phenotype. It is interesting to note that several of them are putatively involved in the oxidative stress response (superoxide dismutases (SOD) [33; 34; 35], thioredoxins (TRX) [36], glutathione-S-transferases (GSTs) [37; 38; 39].

Eighteen probes did not hybridize to the RNA target. A large portion of those probes (38%), belong to the COE group with 28% from the P450 family. This could be due to

the reduced sequence similarity between *An. gambiae* and *An. funestus* and also these genes may be present in the larval stages of the mosquito life cycle and not in the adult stage. Surprisingly two P450 genes, *CYP6P4* and *CYP6P1*, which were significantly differentially expressed in a study by Wondji *et al.* [19], did not hybridize to the array. The *CYP6P1* and *CYP6P4* genes have been shown to have amino acid identities to that of *An. gambiae,* 82% and 89% respectively on small partial sequences analysis [18]. It is unknown why these genes did not hybridize to the slide. This needs to be investigated further.

5. Conclusions

The *An. gambiae* 'detox chip' provided valuable information in identifying potential genes involved in insecticide resistant in *An. funestus*. We acknowledge the fact that heterologous hybridization is most likely an under representation of the detoxifying genes that are differentially expressed, but it is currently the only tool available to use to screen multiple probes at once. This is a useful and an inexpensive alternative given that the development of a species-specific array will require genome sequencing of the specific species to be investigated and a *An. funestus* genome was not available at the time. This information will ultimately be used to understand the underlying mechanisms involved in resistance of this important malaria vector and hence aid in the control of effective resistant management.

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