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Genetic and biochemical characterization of the cytochrome P450, CYP6P9, associated with pyrethroid resistance in the African malaria vector Anopheles funestus

Stradi, Melanie

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CHAPTER 3

Investigating CYP6P9, CYP6P13 and Cytochrome Oxidase I (COI) gene transcription post pyrethroid (deltamethrin) exposure

3.1 Introduction

CYP6P9 and CYP6P13 are two duplicated P450 genes sharing 93.7% sequence similarity between them (Wondji *et al.,* 2009; Matambo *et al*., 2010). CYP6P13 was most likely due to a gene duplication event of CYP6P9 (Wondji *et al.*, 2009). Wondji *et al*. (2009) named this duplicate gene CYP6P9b, while Matambo (2008) and Matambo *et al.* (2010) named this gene CYP6P13 based on naming from the P450 nomenclature committee (http://www.cytochromep450.net/cytochrome-p450-nomenclature-committee) without prior knowledge of the work done by Wondji *et al.* (2009). The sequence similarities between CYP6P9b and CYP6P13 indicated that these genes are the same. In this dissertation the gene naming of Matambo *et al.* (2010) will be used.

Higher levels of CYP6P9 and CYP6P13 mRNA were found in pyrethroid-resistant *An. funestus* strains when compared to pyrethroid-susceptible *An. funestus* (Amenya *et al*., 2008; Wondji *et al*., 2009, Morgan *et al*., 2010; Cuamba *et al*., 2010). Both these genes were also found to be overexpressed in *An. funestus* (FUMOZ-R) in a recent microarray study by Christian *et al*. (2011). Increased levels of P450s and specifically the overexpression of both CYP6P9 and CYP6P13, observed by qPCR have also been identified in wild *An. funestus* from Mozambique (Tihuquine) (Cuamba *et al*., 2010), suggesting that the main mechanism of pyrethroid resistance in this population is also elevated levels of P450 enzymes. As both these genes have been implicated in playing a role in insecticide resistance in *An. funestus*, it was of

interest to determine if pyrethroid (deltamethrin) exposure further induced the transcription of these two genes, CYP6P9 and CYP6P13.

In addition to the above mentioned genes, a recent microarray study found insect mitochondrial cytochrome c oxidase subunit I (COI) gene to be differentially expressed (2.7-fold) in *An. funestus* strains (Christian *et al.*, 2011). However when the microarray results were validated using qPCR, COI was not significantly different between the resistant and susceptible strains. It was therefore decided to include this gene in this study. COI is involved in oxidative phosphorylation, suggesting an enhanced mitochondrial gene expression to improve energy supply (Fernandez *et al.*, 2010). It is a conserved region within the genome and is the most slowly evolving region of all mitochondrial protein-coding genes (Mohanty *et al.*, 2009). Cytochrome oxidase is comprised of two catalytic subunits, I and II. Subunit I possesses features suitable for evolutionary studies (Lunt *et al*., 1996) and is a large and highly hydrophobic protein encoded in the mitochondrial genome (Fontanesi *et al*., 2008). Subunit II (COII) transfers the electrons from cytochrome c to the catalytic subunit I (Speno *et al.*, 1995). It provides the substrate-binding site and contains a Cu (A) centre that is probably the primary acceptor in cytochrome c oxidase (Capaldi *et al*., 1983).

Changes in the transcription level of genes involved in energy metabolism, such as COI, may influence important traits (Roberge *et al*., 2007). The provision of ATP and the control of its metabolism seem to be critical components of the general environmental stress response in all organisms, allowing them to respond with adaptive physiological changes while, at the same time, buffering the changing energy demands (Gracey and Cossins, 2003). Revealing the functional importance between gene transcription and exposure to pyrethroids in *An. funestus*,

may help elucidate COI's role and significance in detoxification. The effect of pyrethroid exposure on the transcription of three genes, CYP6P9, CYP6P13 and COI, has not been investigated in *An. funestus*.

3.2 Materials and methods

3.2.1 *Biological samples and total RNA isolation*

FUMOZ-R 3-day-old females were exposed to deltamethrin (0.05%) insecticide for 1 hour and then used for analysis or exposed for one hour followed by a two hour recovery period before RNA extraction. RNA was extracted from both cohorts and converted to cDNA and levels of expression for each gene (CYP6P9, COI and CYP6P13) were quantified using Real-time PCR, against Ribosomal protein Subunit 7 [Rps 7 (S7)] (housekeeping gene) as reference. Three biological replicates (each replicate comprised 15 pooled females) were used and each biological repeat was performed in triplicate (i.e. three technical repeats).

Total RNA was extracted using Tri-reagent (Sigma-Aldrich, T9424-200ml, South Africa) according to manufacturer's instructions, and contaminant genomic DNA removed by DNase I (RNase-Free Dnase Set (50) cat no: 79254, Qiagen, South Africa) treatment. This involved heat killing DNase I at 70 °C for 15min and then placing on ice for 5min. All plasticware, glassware, pipettes and filter tips were cleaned with RNaseZap® (cat no: AM9780, AEC-Amersham Pty Ltd, South Africa) and sterilized under UV light for 2 h prior to use. Briefly, 15 mosquitoes were homogenized in Tri-reagent followed by precipitation using isopropanol with the RNA pellet resuspended in sterile distilled water. The integrity of the RNA was verified by electrophoresis on formaldehyde denaturing agarose gel whilst the total RNA concentration was determined using a Nanodrop® spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and RNA quality verified by A_{260nm}/A_{280nm} ratios > 1.8 (Tables 3.3 and 3.4). This ratio provided an estimate of the purity of the nucleic acid.

3.2.2 *cDNA synthesis and quantitative Real-Time PCR (qRT-PCR)*

Extracted total RNA was reverse transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, 4387406, South Africa) and stored at -70°C. qRT-PCR was carried out using 1µl cDNA [10 nanograms (ng)] and 2.5µM of each degenerate primer: CYP6P9, CYP6P13 and COI (See Table 3.1 for primer sequences). The recommended cycling conditions for each gene can be seen in Table 3.2. All the melt curve temperatures ranged from 40ºC to 95ºC with 1ºC increments for 5 seconds with plate reading. Quantitative analysis of gene expression using qRT-PCR typically requires the use of a constitutively expressed reference gene as an internal control to normalize for differences in starting cDNA template between samples (reviewed in Sturzenbaum and Kille, 2001; Bustin, 2002). The expression level of the reference gene Rps 7 (S7) (GenBank: EF450776) was used as an internal reference (Christian *et al.*, 2011). The detection system entailed the use of IQ™ SYBR Green I supermix (Bio Rad, 1708882, South Africa) fluorescent dye. No template controls for each of the genes was used. S7 was chosen as it was the most stable reference gene, compared to beta-actin, 18S, TATA-binding Protein (TBP) and GAPDH, which were all investigated with the samples under investigation.

Table 3.1 Primers used for qRT-PCR			
Name	Forward primer	Reverse primer	Tm (°C)
CYP6P9[a]	5'-AGA TGT GAT TGG CAC CTG T-3'	5'-TCG ATA TTC CAC CGT TTC CT-3'	82
CYP6P13[a]	5'-CTG GAT CTC CTA ATT ATG ATG AAG TTT TTC -3'	5'-GTT CAC CGT CTC GCG GAC T-3'	64
COI[a]	5'-ATG GAG CAG GAA CAG GAT GAA CAG-3'	5'-AAT CAA CTG AAG CAC CAG CAT GAG-3'	63
Rps 7[b]	5'-TTA CTG CTG TGT ACG ATG CC-3'	5'-GAT GGT GGT CTG CTG GTT C-3'	85

[a] Christian *et al*. (2011)

[b] Amenya *et al*. (2008)

Table 3.2 Cycling conditions for each gene						
Cycling conditions	CYP6P9[a]		CYP6P13[a]		COI[a]	
	Temp (°C)	Time	Temp (°C)	Time	Temp (°C)	Time
Initial denaturation	95	3 min	94	2 min	94	2 min
Cycling (39X)	95	10 sec	94	30 sec	94	30 sec
	55	15 sec	59	30 sec	56.6	30 sec
	72	15 sec	72	40 sec	72	40 sec
Final extension	72	30 sec	72	5 min	72	10 min
Post-PCR melt curve analysis for all genes	40ºC to 95ºC with 1ºC increments for 5 seconds with plate reading.					

[a] Christian *et al*. (2011)

3.2.3 Quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR was performed using the BioRad CFX96™ Real-Time PCR Detection System (Bio Rad, Hercules, CA, USA). Standard curves were generated for each transcript tested using a 2-fold serial dilution of *An. funestus* FUMOZ-R cDNA ranging from 2000ng to 0.98ng per

reaction. All reactions were performed in triplicate in a total volume of 25µl containing 12.5µl of SYBR Green PCR Master Mix and 400nmol of each primer at the conditions mentioned in Section 3.2.2. Three biological replicates were used and for each biological sample, three technical repeats were performed for each gene of interest including the reference gene. PCR results were analyzed using a standard curve. The slope of the standard curve was used to validate the reaction and samples that did not show the recommended slope (-3.3 to -3.8) or PCR efficiency (between 95-100%) were discarded from analysis [Critical factors for successful qPCR (http://www.Qiagen.com)]. The type of analysis used was that of relative quantification (fold expression). The fold-over expression values were determined using the method outlined by Pfaffl (2001), whereby ΔCT values for target and reference genes are calculated and the relative expression ratio between the target and reference genes is determined taking the amplification efficiency into account. Raw quantification cycle (Cq) values were used in these calculations.

3.2.4 Denaturing agarose gel electrophoresis of RNA

Preparation of the 1.4% formaldehyde gel

The 1.4% formaldehyde gel was prepared as follows, modified from the method by Schneider (2002) (http://euphrates.wpunj.edu/courses/genex/Northern.html): 0.7g agarose and 44ml DEPC-treated water was mixed together and heated till all agarose dissolved and then allowed to cool to 55-60°C. 10X MOPS [3-(N-morpholino) propanesulfonic acid] formaldehyde gel running buffer (5ml), 37% formaldehyde solution (1.5ml) and ethidium bromide (3µl) was added to the agarose and swirled gently to prevent air bubbles from forming. As RNA is susceptible to alkali cleavage at high pH, MOPS was chosen due to its high buffering capacity at pH 7.0 and formaldehyde was included in the running buffer to keep the RNA denatured.

The gel was poured and left to cool for at least 30 min and then pre-run for 15 min at 40V. Whilst this occurred, RNA samples were prepared for electrophoresis.

Preparation of RNA samples for electrophoresis

For each sample, the following was mixed in a 0.5ml RNAse free microcentrifuge tube, modified from Schneider (2002): 10X MOPS running buffer (2.5µl), 37% formaldehyde solution (4.4µl), formamide (12.5µl), RNA (2-10µg; max 5.6µl) and water, to make up a total volume of 25µl. The samples were briefly vortexed and centrifuged prior to incubation for 8 min at 75°C and then immediately placed on ice. Orange-G loading buffer [0.35% (w/v) Orange-G, 30% (w/v) Ficoll 400, 1mM EDTA (disodium; in DEPC-treated water)] (5µl) and ethidium bromide (0.15µl; 500µg/ml) was then added to each sample. The RNA marker was treated and prepared the same way as the samples just excluding RNA in the mixture.

Loading of the gel

The gel chamber was filled with 1X MOPS gel running buffer and combs were carefully removed from the gel. The gels were loaded with RNA mix plus ethidium bromide and electrophoresed at 5-5V/cm for 45 min until the dye was 2/3 through the gel. The gel was then removed from the gel-box and visualized on a UV transilluminator to detect for 28S and 18S bands.

3.3 Results and discussion

Real-time PCR is a technique used to quantify gene expression, which correlates PCR product concentration to fluorescence intensity, in real-time (Wong and Medrano, 2005; Higuchi *et al.*, 1993). Real-time PCR is one of the most sensitive, efficient, reproducible and reliably quantitative methods for gene expression analysis (Yuan *et al.*, 2006). There are two methods of real-time PCR used for quantifying mRNA, namely one-step and two-step real-time PCR. In the one-step reaction, the entire reaction from cDNA synthesis to PCR amplification occurs in a single tube, whereas in the two-step reaction, reverse transcription and PCR amplification occurs in separate tubes (Wong and Medrano, 2005). The two-step reaction is generally preferred when using a DNA binding dye such as SYBR Green I, as it is easier to eliminate primer-dimers through the manipulation of melting temperatures (T_ms) (Vandesompele *et al*, 2002a), however there is increased opportunity for DNA contamination.

Low efficiency reactions (<90%) may be caused by contaminating Taq inhibitors, high or suboptimal annealing temperature, old or inactive Taq, poorly designed primers, or amplicons with secondary structures (Taylor *et al.*, 2010). High reaction efficiency (>110%) is generally the result of primer-dimers or nonspecific amplicons (Taylor *et al.*, 2010). Although most runs are reproducible in a two-step reaction, there is variability in the results observed, generally caused by three factors namely biological variability, technical variability and inappropriate experimental design (Bustin, 2010). Biological variability defines that of genetic and phenotypic variation, which also depends on its environmental interactions, as well as the intrinsic stochastic kinetic noise of biochemical reactions (Bustin, 2010; Raser and O'Shea, 2005). Stochasticity in gene expression (Raj *et al.*, 2006) results in genetically identical cells exposed to the same conditions showing significant variation in both their

mRNA (Peixoto *et al.*, 2004; Bengtsson *et al.*, 2005) and protein (Cai *et al.*, 2006) expression patterns, resulting in marked differences in phenotypic characteristics (Kaern *et al.*, 2005; Bahar *et al.*, 2006; Bustin, 2010). Due to this, any conclusions must be qualified by placing them into specific experimental contexts such as age, time, gender, tissue etc. Our samples under investigation were classified according to age (3-day-old), gender (females), and strain (FUMOZ-R), and were subjected to the experimental treatment of 1hr deltamethrin (0.05%) exposure.

Reference genes used for RT-qPCR experiments are used to normalize data by correcting for differences in quantities of cDNA used as a template (Gutierrez *et al.*, 2008; Huggett *et al.*, 2005; Vandesompele *et al.*, 2002b). Reference genes like glyceraldeyde-3-phosphate dehydrogenase (GAPDH), albumins, actins, tubulins, cyclophilin, 18S ribosomal RNA (rRNA) or 28S rRNA are generally applicable (Pfaffl, 2001). The reference gene expression should not vary between the cells or tissues under investigation, in response to any experimental treatment. For this reason, finding a suitable reference gene is vital for correct gene expression analysis.

Real-time PCR can be quantified both absolutely and relatively. Absolute quantification employs an internal or external calibration curve to derive the input template copy number. Relative quantification compares the expression of a target gene to a reference gene and the expression of the same gene in target sample versus reference samples (Pfaffl, 2001). Relative quantification was applicable to this study as all target genes tested (CYP6P9, CYP6P13 and COI) were compared to the reference gene Rps 7 (S7). The Pfaffl model (Pfaffl, 2001) was

used to calculate the fold-over expression. This model incorporates the amplification efficiencies of the target and reference genes to correct for differences between the two assays.

3.3.1 Assessing the total RNA integrity used for qRT-PCR reactions

The integrity of extracted RNA is commonly assessed by gel electrophoresis by analysis of ribosomal RNA (rRNA) fragments, which helps verify the mRNA expression profiling results obtained from quantitative RT-PCR. In mosquitoes, each rDNA transcriptional unit is composed of an External Transcribed Spacer (ETS), an 18S gene, an Internal Transcribed Spacer I (ITS1), a 5.8S gene, an ITS2, and a 28S gene (Paskewitz *et al*., 1993; Hackett *et al*., 2000). In eukaryotes, an intact sample is generally defined as displaying clear and distinct bands for both the 18S and 28S ribosomal subunits, with the 18S band normally displaying twice the strength of the 28S band. If this integrity is maintained, it is assumed to reflect the integrity of other fractions of RNA, for example the mRNA fraction in the case of expression studies (Winnebeck *et al.*, 2010). Additionally, the purity of RNA is indicated by A260/A280nm values showing values ≥ 1.8. This is indicated in Table 3.3 and Table 3.4.

Degradation of total RNA can be observed by smears on a formaldehyde gel (Figure 3.1). RNA degradation could be a result of ribonucleases in the electrophoresis buffer or tank that may still have been present even after cleaning (Bryant and Manning, 2000); therefore care must be taken to ensure that the equipment is clean and that both this and the buffer are nuclease free. Lanes 1-3 (Figure 3.1) are indicative of RNA with good integrity that can be used for qRT-PCR experiments and lanes 4-7 (Figure 3.1) are indicative of degraded RNA samples that are not used for qRT-PCR experiments.

Results (Figure 3.1) showed a single 18S band, instead of both 18S and 28S. This phenomenon is commonly found in insect RNA (Winnebeck *et al*., 2010). The results from the *An. funestus* RNA could reflect what is seen in the *Drosophila* species – whereby *Drosophil*a 28S rRNA is processed into two fragments that migrate in a similar manner to the 18S rRNA (Ambions online appendix – ribosomal RNA sizes: http://www.ambion.com/techlib/append/ribosomalRNAsizes.html). Winnebeck *et al.* (2010) states that the 28S rRNA of most insects contains an endogenous 'hidden break', which upon heat-denaturation, disrupts the hydrogen bonds leading to the release of two similar sized fragments that both migrate closely with the 18S rRNA. When samples are not heat-denatured, both bands are clearly visible. This 28S rRNA thermolability is not only confined to Diptera and Lepidoptera; other insects such as *Apis mellifera* (Hymenoptera) (Shine and Dalgarno, 1973; De Lucca *et al.*, 1974; Gillepsie *et al.*, 2006) display this phenomenon too, along with other protostomes (Ishikawa, 1977) and in several South American rodent species of the genus *Ctenomys* (Melen *et al.*, 1999; Winnebeck *et al.*, 2010).

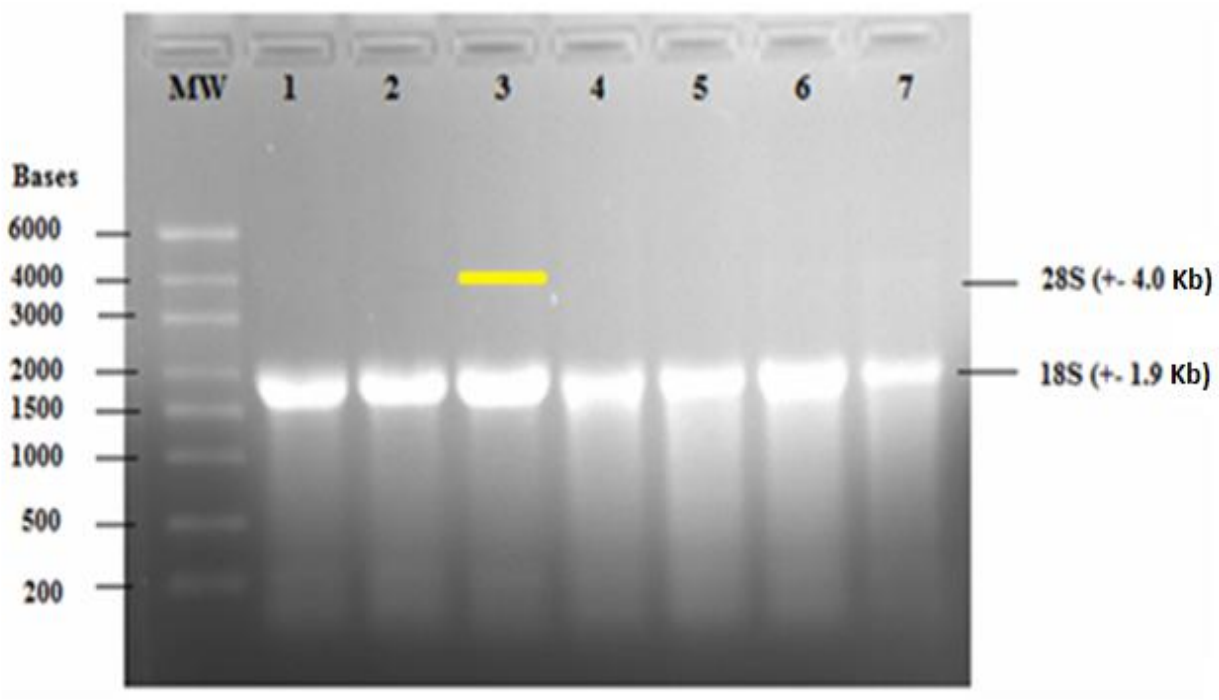

Figure 3.1: Example of intact and degraded total RNA run beside an RNA ladder on a 1.4% formaldehyde gel. The 18S ribosomal RNA fragments are clearly visible in the RNA samples from *An. funestus* (FUMOZ-R), however the 28S rRNA fragment (around 4Kb) is not visible due to an endogenous 'hidden break', as explained by Winnebeck *et al.* (2010). **Lanes 1 – 3:** Showing RNA extractions with good integrity. **Lanes 4 - 7:** degraded RNA that cannot be used for cDNA synthesis; **MW = Molecular weight marker/RNA ladder.** The inserted yellow 4.0Kb line indicates where the 28S fragment would normally be seen for all samples tested.

Table 3.3 RNA nanodrop readings (A260nm/A280nm) for all samples under investigation				
		A260nm/A280nm value ≥ 1.8		
Sample (FUMOZ-R females; n = 15)	**Biological Replicate**	**Technical repeat 1**	**Technical repeat 2**	**Technical repeat 3**
Untreated/unexposed	**1**	**1.98**	**2.0**	**1.98**
	2	**1.90**	**1.88**	**1.85**
	3	**1.90**	**1.85**	**1.87**
1hr deltamethrin (0.05%) exposure	**1**	**2.0**	**2.06**	**2.01**
	2	**1.72***	**1.85**	**1.92**
	3	**1.82**	**1.83**	**1.87**
1hr deltamethrin (0.05%) + 2hr recovery	**1**	**1.93**	**1.91**	**1.90**
	2	**1.86**	**1.84**	**1.85**
	3	**1.91**	**1.87**	**1.83**
Note: Blank = deionised water, Absorbance = 0				

*** Only sample to show A260/A280nm value less than 1.8**

Table 3.4 RNA nanodrop readings for FUMOZ-R mixed samples (male and female) used in Standard Curve			
Sample	**ng/µl**	**Absorbance**	**A260/A280nm value**
1	**1271.5**	**49.480**	**1.89**
2	**1141.8**	**41.543**	**1.90**
3	**1456.3**	**13.517**	**1.91**
4	**1270.4**	**43.764**	**1.90**
5	**1164.1**	**45.418**	**1.90**
6	**1249.3**	**43.513**	**1.88**
7	**1228.8**	**49.563**	**1.88**
8	**1618.6**	**52.624**	**1.91**
9	**1424.2**	**54.673**	**1.88**
10	**1029.7**	**37.975**	**1.88**
11	**1232.8**	**48.457**	**1.88**

3.3.2 CYP6P9, CYP6P13 and COI transcription profiling analysis

In order to study the change in expression levels of CYP6P9, CYP6P13 and COI in the *An. funestus* resistant strain (FUMOZ-R) exposed to pyrethroids, quantitative real-time PCR was chosen above other techniques due to its sensitivity, as the amplicons are visualized during the exponential phase. Multiple reference genes were assessed for use as an internal control in this study. The genes chosen for comparison with CYP6P9, CYP6P13 and COI were Rps 7 (S7), ribosomal subunit 18S, GAPDH, beta-actin, and TATA-binding protein (TBP). Evaluation and validation of the reference genes detected large biological variation between the exposed versus unexposed samples directly after 1hr exposure to pyrethroids, but this was not detected when samples were left to recover for 2hrs prior analysis (Figure 3.2). Due to this reason the 1hr exposed samples were not analysed further. The most suitable reference gene was S7 (Figure 3.3) whilst beta-actin and TBP did not amplify, very likely because these primers were designed against *An. arabiensis* and not *An. funestus*. The instability of these reference genes following 1hr exposure to deltamethrin (pyrethroid) would need to be investigated in future studies. It is postulated that due to the stress of insecticide exposure, P450 genes including reference genes such as S7, 18S and GAPDH, are induced to ensure the survival of the mosquito.

Previous qPCR studies from Amenya *et al*. (2008) and Christian *et al.* (2011) used S7 and it was therefore decided to use the same reference gene. As CYP6P9 expression is not sex specific as observed by experiments performed by Amenya *et al*. (2008), only 3-day-old females were used in this analysis for the deltamethrin treatments, and both males and females were used for creating the standard curve for all comparisons.

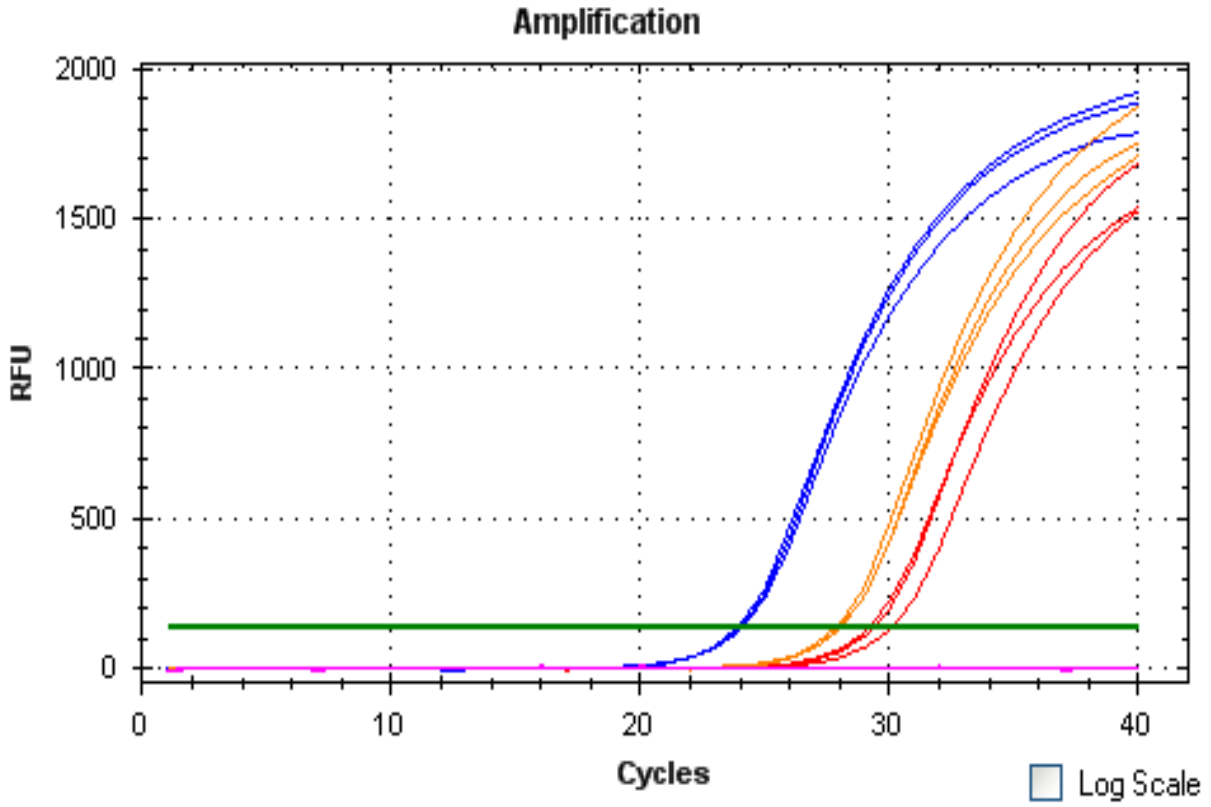

Figure 3.2: Amplification profile of GAPDH and 18S showing differences in Cq-values for both 1hr deltamethrin (blue) and 1hr plus 2hr recovery (orange) treatments compared to the unexposed (red), making it unsuitable to use as a reference gene. The no template control (NTC) is indicated in pink. No amplification was detectable in the absence of template. X-axis = quantification cycle (C_q); Y-axis = relative fluorescence units (RFU).

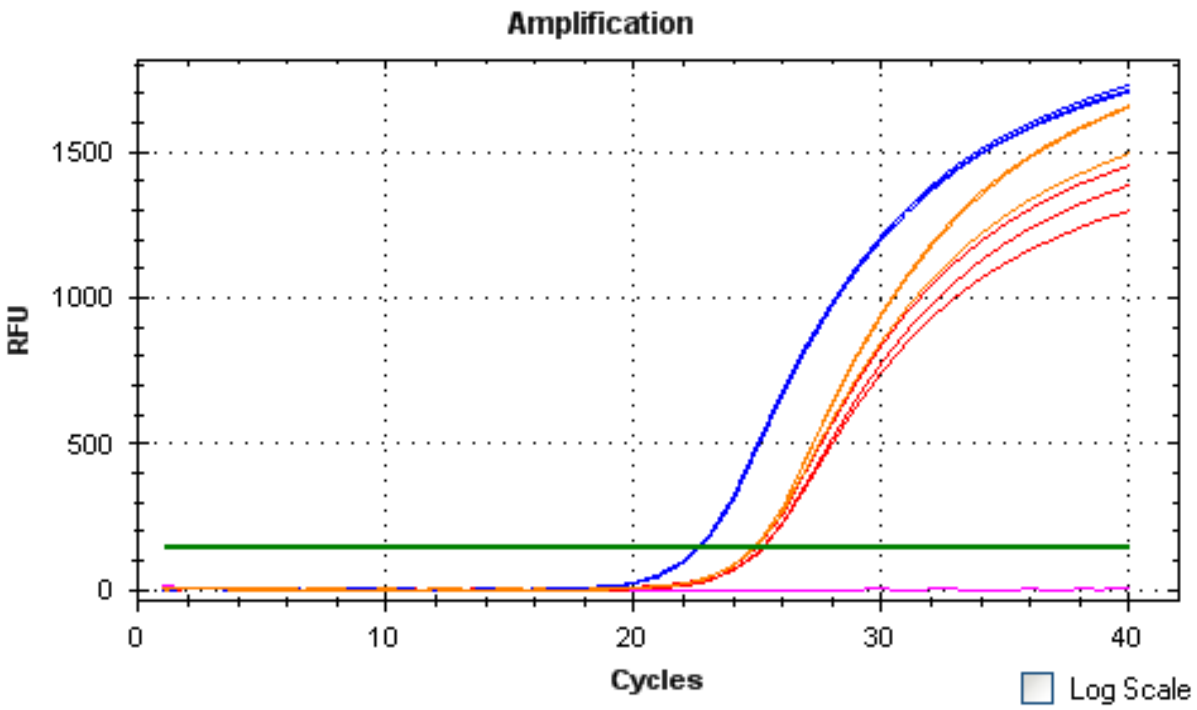

Figure 3.3: S7 amplification profile when run with CYP6P9, CYP6P13 and COI amplification protocols; showing unexposed (red), 1hr deltamethrin (blue), and 1hr plus 2hr recovery (orange). The no template control (NTC) is indicated in pink. The 1hr deltamethrin treatment is upregulated by approximately 2 quantification cycles (Cq). All treatments fell within the standard curve. X-axis = quantification cycle (C_q); Y-axis = relative fluorescence units (RFU).

When exposing 3-day-old FUMOZ-R females to 0.05% deltamethrin, induction of the reference genes (S7, 18S, GAPDH) occurred as described earlier. However, upon 1hr deltamethrin exposure and a 2hr recovery period, the FUMOZ-R females' gene transcription recovered to nearly resemble that of the untreated (unexposed) state for all 3 target genes tested (Figure 3.3). Relative quantification analysis revealed that the CYP6P9 gene had a 1.56-fold (± 0.10) increase in transcription, while CYP6P13 and COI had a 2.52-fold (± 0.16) and 2.72-fold (± 0.14) increase respectively (Figure 3.4). Mean Cq-values [previously known as Ct (threshold cycle)] and standard deviations are represented (Figure 3.4). CYP6P9 showed

to be the least affected by deltamethrin exposure and this is illustrated by a small 1.56-fold difference. However, the gene transcription is still increased slightly. Although CYP6P13 has been identified as a duplicate gene of CYP6P9, the fold over-expression of 2.52 is much higher than that of CYP6P9. Genes were considered to be differentially expressed if they demonstrated a ≥1.5-fold change in expression between them and a value of $P<0.05$. Statistical analysis revealed there was no significant difference between these three genes, calculated using the two-sample Student's *t*-test assuming equal variances and 95% confidence intervals (CIs), $P<0.05$ (Statistix 7, Analytical software version 7.0).

Christian *et al.* (2011) found that resistant (FUMOZ-R) males showed a fold change of 51 for the CYP6P9 gene, 15 for CYP6P13 and one for COI when compared to the susceptible strain after qPCR analysis was performed. The females also showed the highest fold change in CYP6P9 (67), followed by CYP6P13 (8) and COI (4) (Christian *et al.*, 2011). In addition to this high level of mRNA expression, it was surprising that this mRNA expression could be further induced in the resistant strain by exposure to deltamethrin. Differences in the transcription of CYP6P9 and CYP6P13 in response to deltamethrin exposure might indicate that they are not under the same transcription regulation system. This would need to be investigated further before any further conclusion can be made.

Interestingly, difference in fold over-expression between resistant and susceptible adults of CYP6P9 has been reported by Cuamba *et al.* (2010) and Morgan *et al*. (2010). CYP6P13 was consistently expressed at a higher ratio than CYP6P9 with respectively a 15-fold and 12-fold overexpression (Cuamba *et al.*, 2010) and the CYP6P13 copy was 12 times overexpressed in females from Tororo compared to females of the susceptible FANG strain and 11-times for the

males (Morgan *et al.*, 2010). The results presented here and those from Cuamba *et al.* (2010) and Morgan *et al*. (2010) shows some of the complexities in this newly characterized resistance mechanism. Different pyrethroid resistant *An. funestus* populations seem to have some similarities, but one mechanism is not a duplication of the other.

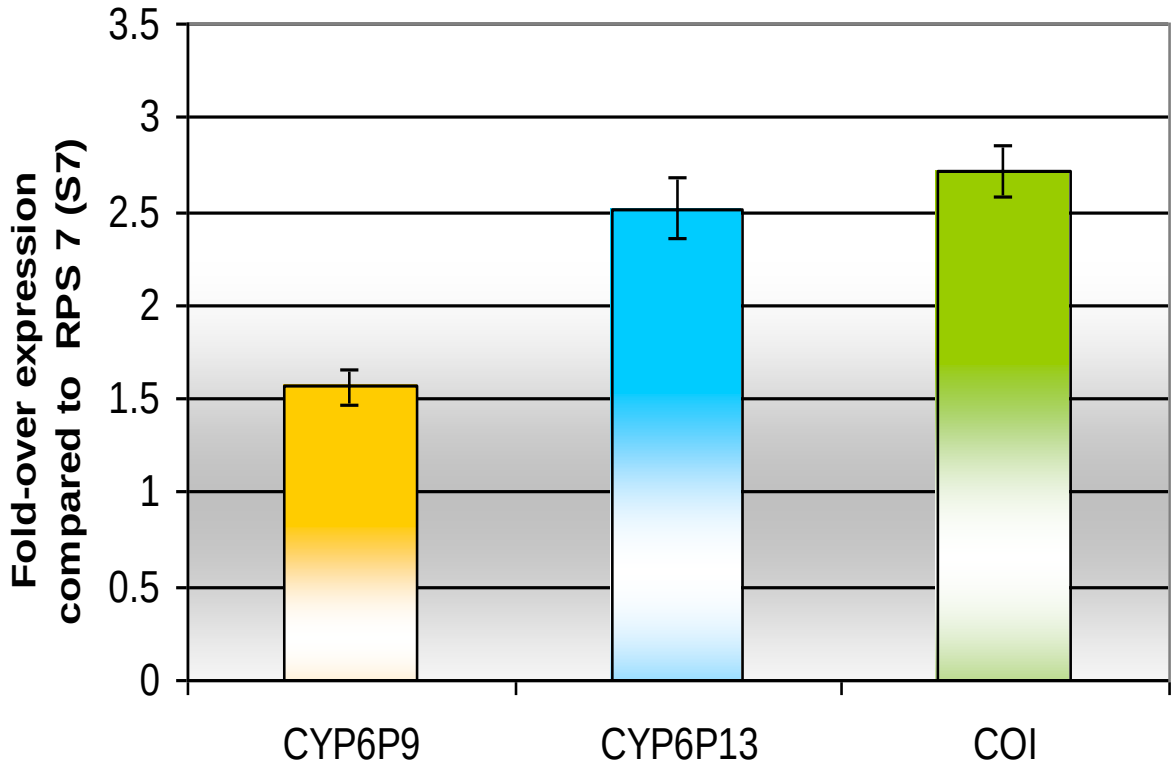

Figure 3.4: Relative fold-over expression levels of CYP6P9, CYP6P13 and COI genes 2hr post deltamethrin exposure. The normalized expression ratio of each gene against S7 gene is represented on the vertical axis. Fold-over expression values are as follows: **CYP6P9 = 1.56 (± 0.10); CYP6P13= 2.52 (± 0.16); COI = 2.72 (± 0.14)**. Three biological replicates consisting of 15 pooled female mosquitoes per replicate were used and each biological replicate was performed in triplicate.

COI gene transcription was expressed 5-10 (Cq) cycles earlier than CYP6P9 and CYP6P13, which indicates that there is more mRNA present than in the other two genes. COI showed the highest fold overexpression of 2.72, although it was not previously shown by qPCR to be significantly upregulated in the resistant strain (Christian *et al*., 2011). As COI is a mitochondrial gene that works to supply the cells with ATP for energy, it is postulated that an increase in COI transcription may be needed to supply the energy needed for other downstream pathways and genes involved in detoxification. Further investigations are needed to verify the exact role COI could play alongside these P450s.

Reproducibility of qRT-PCR was tested for CYP6P9. Three replicate analyses on another machine, the Light Cycler® 480 (LC480) was conducted. All reaction conditions remained similar (Tables 3.1 and 3.2) except for the 96-well plates which were white in colour (compared to clear plates used on the Bio Rad CFX96^{TM} System). This is important because different plastics exhibit substantial differences in fluorescence reflection and sensitivity (Reiter and Pfaffl, 2008). The dissociation or melt curve conditions were changed to 1 degree increments for 1 second, and not 5 seconds (Table 3.2). Pfaffl (2001) analyses were carried out on the data and compared to our previous data. It was found that the fold over-expression for CYP6P9 (2hr post exposure) on the LC480 system was 1.59-fold overexpressed. This compares very well with the 1.56-fold over-expression reported from the Bio Rad CFX96^{TM} System. There was no statistical difference ($P<0.05$) between the data performed on the two machines. Results of amplification curves, standard curves and melting peaks performed on the LC480 can be viewed in Appendix A2.

3.4 Conclusion

Anopheles funestus FUMOZ-R CYP6P9 mRNA showed a small increase, only 1.56-fold in expression, compared to unexposed samples. CYP6P9 and CYP6P13 displayed different transcription profiles in response to deltamethrin exposure which may indicate that they are not under the same transcription regulation system. Different pyrethroid resistant *An. funestus* populations display some similarities in gene transcription however; one mechanism is not a duplication of the other. Over-expression of P450 genes, and induction of genes especially from the CYP6 family, is an adaptive or even possibly a genetic ability for metabolic detoxification of pyrethroid (deltamethrin) insecticides in *An. funestus*. Deltamethrin exposure leads to increased transcription of CYP6P9, CYP6P13 and COI. These transcription results provide further support for the role of CYP6P9 and CYP6P13 in conferring pyrethroid resistance in *An. funestus*.