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THE EFFECTS OF PYRETHROID RESISTANCE ON TRANSCRIPTION OF METABOLIC ENZYMES IN A MAJOR AFRICAN MALARIA VECTOR, *ANOPHELES FUNESTUS*

Riann N. Christian

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of Doctor of Philosophy

Johannesburg, 2011

DECLARATION

I, Riann Christian, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……. (Signature of candidate)

25 Day of May, 2011

DEDICATION

To my loving husband Rolan and my parents.

Also in loving memory of Julian Naguran.

PUBLICATIONS AND PRESENTATIONS:

Publications:

Christian, R. N., T. S. Matambo, B. L. Spillings, B. D. Brooke, M. Coetzee, and L. L. Koekemoer. 2011. Age-related pyrethroid resistance and P450 gene expression in the major African malaria vector, *Anopheles funestus* (Diptera: Culicidae). Accepted by Genet Mol Res (Appendix C).

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Christian, R., L. L. Koekemoer, H. Ranson, and M. Coetzee. 2006. Microarray analysis of gene expression in the major malaria vectors (*Anopheles funestus* and *An. arabiensis*) resistant to insecticides. Poster presentation. MCBG symposium, Johannesburg, South Africa, 5 October.

Christian, R., T. S. Matambo, B. L. Spillings, B. D. Brooke, M. Coetzee, and L. L. Koekemoer. 2008. The effects of pyrethroid resistance on transcription of metabolic enzymes in a major African malaria vector, *Anopheles funestus*. Talk. 23rd International Congress of Entomology, ICC, Durban, South Africa, 6-12 July.

Christian, R., L. L. Koekemoer, H. Ranson, and M. Coetzee. 2009. Microarray analysis on the major malaria vectors (*Anopheles funestus* and *An. arabiensis*)

resistant to insecticides. Poster presentation. Bioinformatics and Genomics approaches for functional gene expression in vector-virus-host interactions, WITS University, Johannesburg, South Africa, 30-31 March.

Christian, R., T. S. Matambo, B. L. Spillings, B. D. Brooke, M. Coetzee, and L. L. Koekemoer. 2009. Cytochrome P450 genes, (*CYP6P9* and *CYP6P13*) overexpressed in pyrethroid resistant *Anopheles funestus* (Diptera: Culicidae). Talk. 16th Entomological Congress, Stellenbosch, South Africa, 5-7 July.

ABSTRACT

Anopheles funestus is a major vector of malaria in the southern African region. Insecticide resistance to pyrethroid and carbamate insecticides has been recorded in populations of this species in South Africa and Mozambique. This study aimed to determine the relationship between pyrethroid resistance and gene expression of two closely related genes, *CYP6P9* and *CYP6P13*, by age and sex in a resistant strain *An. funestus* from southern Africa, FUMOZ-R. The insecticide susceptibility assays showed that percentage survival in both FUMOZ-R sexes significantly decreased as age increased. The mRNA expressions of *CYP6P9* and *CYP6P13* were higher in FUMOZ-R relative to the insecticide susceptible strain (FANG). The expression of permethrin resistance varies with age in *An. funestus* FUMOZ-R. The results indicate that other genes may also be involved in insecticide resistance. In addition to this, the expression profile of other metabolic genes involved in insecticide resistance was also investigated. A microarray based approach was used to identify genes differentially expressed in FUMOZ-R and FANG. 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*. After optimization of the hybridisation conditions, over 90% of the probes showed a positive signal. Only three genes were significantly (P<0.001) differentially expressed in the females, *CYP6P9*, *COI* and *CYP6M7*. The same genes were also significantly differentially expressed in the adult males, together with an additional 21 transcripts. The third part of this study investigated the gene expression in the first instar, fourth instar and 3-day old adults in FUMOZ-R using the *An. gambiae* detox chip. The variation in metabolic enzyme gene transcription at the different developmental stages in *An. funestus* are not known. The identification of differentially transcribed genes at the

different life stages provides some insight into the role and function of these genes. A large number of cytochrome P450s (monooxygenases), esterases, glutathione *S*-transferases (GSTs) and other additional genes were differentially expressed in all life stages. This study provided vital information regarding genes potentially involved in pyrethroid resistant and is the first to provide metabolic or detoxifying transcription gene information in *An. funestus*.

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ABBREVIATIONS AND SYMBOLS

AchE	acetylcholinesterase
AMP	ampicillan
amRNA	amplified RNA
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	complementary deoxyribonucleic acid
C_Q	quantification cycle
ddH_2O	double distilled water
DDT	dichlorodiphenyltrichloroethane
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
ds	double stranded
dTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FANG	*Anopheles funestus* from Angola
FUMOZ-R	resistant *Anopheles funestus* from Mozambique
Fwd	forward
GABA	γ-Aminobutyric acid
GST	glutathione *S*-transferases
IPTG	isopropyl-β-D-thio-galactoside
IRS	indoor residual spraying
ITN	insecticide treated net
IVM	integrated vector management
IVT	*in vitro* transcription
kdr	knock down resistance
LB	Luria Bertani broth
Limma	Linear Models in Microarray Analysis
mg	milligram
mins	minutes

ml	millilitre
mM	millimolar
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
ng	nanogram
nM	nanomolar
OP	organophosphates
P450	cytochrome P450
PCR	polymerase chain reaction
pg	picogram
pmol	picomole
PMT	photo-multiplier tube
qPCR	quantitative polymerase chain reaction
QTL	quantitative Trait Locus
Rev	reverse
RNA	ribonucleic acid
rpm	revolutions per minute
secs	seconds
SNP	single nucleotide polymorphism
tCNR	transcript copy number
U	Unit
WHO	World Health Organization
XGAL	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
µl	microlitre
µM	micromolar

CHAPTER ONE

INTRODUCTION

There are approximately 3 450 species and sub species of mosquitoes, consisting of 38 genera which all belong to the family Culicidae (Service, 1996). The Culicidae family consists of three sub families, Toxorhynchitinae, Anophelinae (anophelines) and Culicinae (culicines). Mosquitoes are found worldwide in both tropical and temperate regions. They are, however, absent from a few islands and Antarctica (Service, 1996). Mosquitoes are notorious for the spread of various diseases including filariasis, yellow fever, dengue fever, chikungunya, and most importantly malaria.

1.1 Malaria

In 2010, the World Health Organization (WHO) reported that an estimated 765 million people were of risk of malaria. Malaria accounts for 10% of Africa's disease burden and causes the greatest suffering and impoverishment amongst the poor (Figure 1.1).

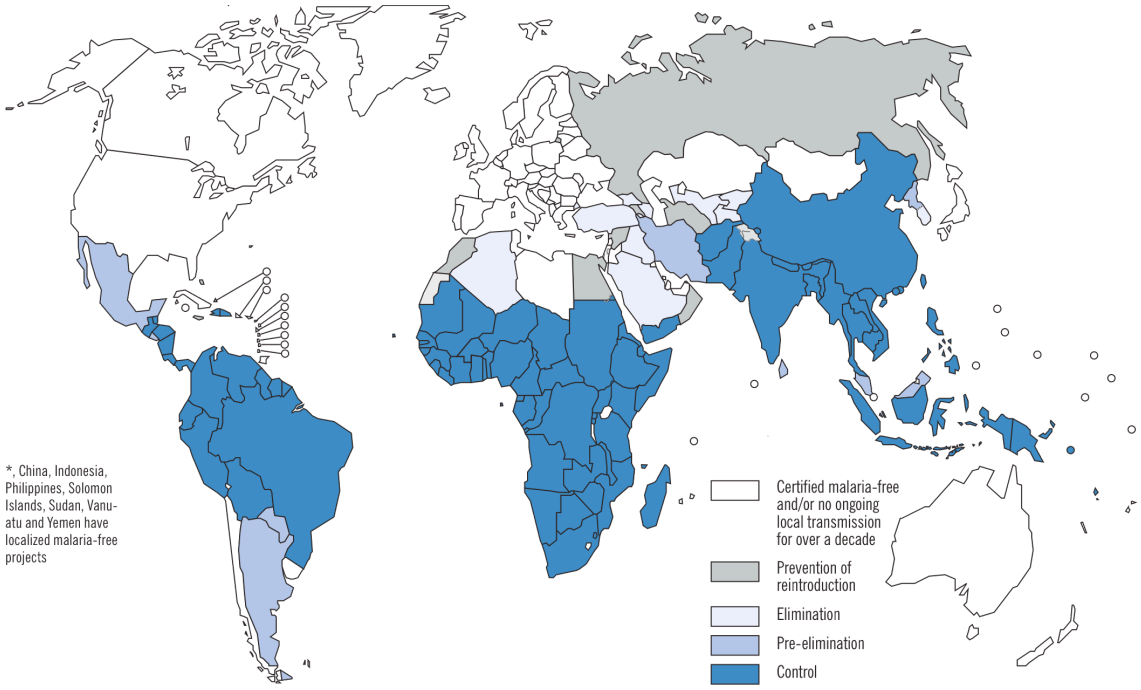

Figure 1.1 Distribution of malaria worldwide and the phases of control in the malarial areas, reported at the end of 2008 (WHO, 2009)

Pregnant women and children under five years of age are usually the most vulnerable (Okenu, 1999; Tren and Bate, 2004). Most malaria deaths occur in Africa and result from both the epidemiological situation on this continent and very limited vector control activities during the past decades. The lack of adequate health services often results in deficiencies in treatment and this is compounded by an increase in drug resistance (WHO, 2004). One of the key components of an effective malaria control programme entails prompt drug treatment and in more severe cases, admission to hospital (reviewed by Hemingway and Bates, 2003). The choice of drug treatment is usually dependant on the parasite resistant patterns and the availability of anti-malarial treatment (www.doh.gov.za).

Malaria control programmes aim to prevent transmission of malaria, aid in prompt clinical diagnosis of malaria infection and provide adequate drug treatment. This leads to reduced

infection as well as morbidity and mortality caused by malaria parasites. The *Plasmodium falciparum* parasite causes about 90% of the malaria cases in most of southern Africa, while the remaining 10% are caused by *P. vivax, P. malariae* and *P. ovale* (Tren and Bate, 2004). These parasites are transmitted to humans by a select few mosquito vectors with the main vectors in Africa, being *Anopheles gambiae* Giles*, An. arabiensis* Patton and *An. funestus* Giles.

1.2 Malaria Vectors

In order to improve our understanding of malaria and the risks involved it is imperative to have knowledge of the biology of the malaria vectors and their distribution.

1.2.1 *Anopheles gambiae* complex

Members of the *An. gambiae* complex are amongst the main vectors of malaria in sub-Saharan Africa (Gillies and Coetzee, 1987). This complex consists of at least seven morphologically similar species, that differ in their ecology, behaviour and feeding (Gillies and De Meillon, 1968; White, 1985; Gillies and Coetzee, 1987; Hunt *et al.*, 1998). The *An. gambiae* complex consists of seven species and these include *An. gambiae s.s., An. arabiensis* Patton, *An. merus* Donitz, *An. melas* Theobald, *An. bwambae* White and *An. quadriannulatus* Theobald species A and B (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Hunt *et al.,* 1998). *Anopheles gambiae* and *An. arabiensis*, two of the major malaria vectors are found in most of Africa (Gillies and Coetzee, 1987). Three of the minor vectors include *An. merus,* a saltwater breeder, found on the eastern side of Africa, *An. melas* found on the west coast and *An. bwambae* found in only one locality in Uganda (Gillies and De Meillon, 1968; White, 1985; Gillies and Coetzee, 1987). *Anopheles*

quadriannulatus, a non-malarial vector, is found in Ethiopia and most of southern Africa (Gillies and Coetzee, 1987; Coetzee *et al.,* 2000).

1.2.2 *Anopheles funestus* group

The third most well known malarial vector, which is the focus of this study, is *An. funestus. Anopheles funestus* mosquitoes are much smaller and darker in colour than the *An. gambiae* complex mosquitoes. The *An. funestus* group historically consisted of nine species with little morphological variation existing between them except at the larval and adult stages. The nine species includes *An. funestus* Giles*, An. rivulorum* Leeson, *An. parensis* Gillies, *An. vaneedeni* Gillies and Coetzee, *An. leesoni* Evans, *An. fuscivenosus* Leeson, *An. aruni* Sobti, *An. brucei* Service and *An. confusus* Evans and Leeson (Gillies and Coetzee, 1987). A new species, *An. rivulorum*-like *sp.* (Cohuet *et al.,* 2003) was identified in Cameroon and it was found to be very similar to *An. rivulorum.* Another new member in this group was recently discovered in Malawi and is morphologically similar to *An. funestus* but different at the genetic level (Spillings *et al.,* 2009). This species, temporarily called *An. funestus*-like, has not been reported to carry *P. falciparum* parasites. Recently, Harbach (2004) restructured the taxonomic classification of the *An. funestus* group. This group now comprises the *An. funestus* subgroup (*An. funestus, An. vaneedeni, An. parensis, An. aruni, An. confusus, An. funestus*-like), the *An. aconitus* subgroup, the *An. culicifacies* subgroup, the *An. minimus* subgroup (*An. leesoni* amongst others) and the *An. rivulorum* subgroup (*An. brucei, An. rivulorum, An. rivulorum*-like and *An. fuscivenosus*).

Anopheles funestus, is highly endophilic and anthropophilic. This implies they feed indoors on humans although alternative hosts might be present (Gillies and De Meillon, 1968).

Anopheles rivulorum, which is usually zoophilic, is the only other species from the *An. funestus* group, that has been implicated in the transmission of malaria in nature (Tanzania) (Wilkes *et al.,* 1996). The other members of this group are zoophilic and do not transmit malaria (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Cohuet *et al.,* 2003). *Anopheles vaneedeni* has been shown to support malaria parasite development under laboratory conditions, but has not been implicated as a malaria vector in nature (De Meillon *et al*., 1977).

1.2.2.1 Distribution

Anopheles funestus, An. rivulorum and *An. leesoni* are widespread throughout sub-Saharan Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). *Anopheles parensis* and *An. confusus* can be found in eastern and southern Africa, *An. aruni* in Zanzibar, *An. fuscivenousus* in Zimbabwe, *An. brucei* in Nigeria and *An. vaneedeni* in the northern areas of South Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The *An. funestus* group generally inhabits areas that have permanent water such as swamps, weedy sides of streams and ponds that are conducive to the breeding of *An. funestus* larvae. *Anopheles funestus* generally prefers water with shade, usually provided by floating vegetation or grass (De Meillon, 1947).

1.2.2.2 Species identification

Anopheles funestus, An. funestus-like*, An. vaneedeni, An. parensis* and *An. aruni* are identical morphologically at all life stages (Gillies and De Meillon, 1968). *Anopheles rivulorum* and *An. brucei* can be distinguished from the other species by their distinctive larvae although they are indistinguishable from each other at the adult stage (Gillies and De Meillon, 1968). *Anopheles leesoni* are distinguished from the other African species by

its early stages (Gillies and De Meillon, 1968) while *An. confusus* is distinguished by its larval characteristics. *Anopheles fuscivenosus* is known only from the adult stage and by chromosomal banding arrangements (Gillies and De Meillon, 1968; Green, 1982).

The identification of malaria vectors is essential for the implementation of vector control strategies. Identification of the different species of the *An. funestus* group is problematic when only adult mosquitoes are available and as a result, molecular methods had to be implemented in order to distinguish between them. In 1980, Green and Hunt conducted a study on the cytogenetic characteristics of *An. funestus*. In 1982, Green reported on *An. funestus* chromosome maps of seven of the species. This was the only genetic method available at the time to distinguish between species. This method is laborious, time consuming and not effective if large samples are to be processed for identification (reviewed by Coetzee and Fontenille, 2004). One of the first molecular studies on *An. funestus* was conducted by Koekemoer *et al.* (1998). In this study, primers were developed in the D3 region in the 28S ribosomal gene. The PCR products were digested which resulted in two fragments that represented *An. funestus* and *An. vaneedeni.* Further studies by Koekemoer *et al.* (1999), used the single-strand confirmation polymorphism technique to identify *An. funestus, An. rivulorum, An. leesoni* and *An. vaneedeni.* This technique was limiting in that only four members of the *An. funestus* group could be identified.

In 2002, Koekemoer *et al.* developed a molecular species-specific polymerase chain reaction (PCR) assay that identifies five members of the *An. funestus* group in Africa, and these include *An. funestus*, *An. vaneedeni*, *An. rivulorum*, *An. leesoni* and *An. parensis*. Another species-specific PCR developed a year later (Cohuet *et al.,* 2003) identified *An.*

rivulorum-like. This species from Cameroon differed genetically from the *An. rivulorum* from South Africa as described in the study conducted by Koekemoer *et al.* (2002).

1.3 Malaria Vector Control

Resurgence of malaria in southern Africa can be attributed to various factors such as: parasite drug resistance, mosquito insecticide resistance, climate change, large scale population migration and the breakdown of vector control programs (reviewed by Booman *et al.,* 2003). The control of malaria cannot be solely by treatment of the disease itself. Both the disease (caused by the parasite) and the vector need to be managed in order to prevent transmission. The control of malaria in most African countries consists of early diagnosis and timeous treatment (Keiser *et al.,* 2004). Most vector control programs use an integrated vector management (IVM) plan. IVM incorporates a wide range of tools such as environmental management (includes larval control), screening of windows and doors, indoor residual spraying (IRS) and insecticide treated bed nets (ITNs) (Okenu, 1999; reviewed by Hemingway and Bates, 2003; Howard *et al.,* 2007). IRS and ITNs are widely used, while other methods mentioned above are used in specific settings.

In Africa, in the 1930's, IRS involved spraying with chemicals such as pyrethrum. Today, pyrethroids are the preferred insecticides of choice due to their low mammalian toxicity and their ability to quickly kill and immobilize insects (Roberts and Andre, 1994; Coetzee *et al.*, 2006). Around the 1950s, this was replaced by dieldrin and DDT (reviewed in Coetzee *et al.,* 1999), which had a much longer life span and therefore had economic advantages. Currently pyrethroids, organochlorines, carbamates and organophosphates are recommended for vector control (WHO, 2008). Despite environmental concerns about

DDT (organochlorines), it is still used for malarial control. The increase in insecticide resistance is of major concern where IRS and ITNs are used as control interventions and insecticide resistance management is imperative. The only insecticide currently allowed for use on ITNs is pyrethroids (reviewed in Coetzee *et al.,* 1999). However, ITNs provide limited protection against mosquitoes biting outdoors and have been perceived as being irrelevant by users (Roberts and Andre, 1994).

The control of malarial vectors is to an extent a countrywide control intervention. Larval control can be achieved by environmental management and community participation. Microbial larvicides have been used and *Bacillus sphaericus* (*Bs*) and *B. thuringiensis var. israelensis* (*Bti*) have shown to give excellent control of the major vectors of malaria in Africa (Fillinger and Lindsay, 2006; Majambere *et al.,* 2007). The use of biological control agents as opposed to chemical larvicides can be attributed to the fact that they are very species-specific and environmentally safe (Majambere *et al.,* 2007). Larvivorous fish have also been used in the control of mosquitoes for decades and have shown to reduce larvae by 75% in ponds (Howard *et al.,* 2007; reviewed by Chandra *et al.,* 2008). However, this method will not be suitable in Africa due to the fact that members of the *An. gambiae* complex breed in temporary pools. Due to the harmful effects of chemicals on mosquitoes and non-target populations and the occurrence of insecticide resistance in mosquito populations, there has been an interest in the use of biological control and biopesticides. The use of entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana* in storage clay pots, have been trialled and tested for use against malarial vectors (Farenhorst *et al.,* 2008). These insect-pathogenic fungi infect insects by penetrating the cuticle, without the need for ingestion, and kill them within a few days (Roy *et al.,* 2006).

Despite the use of biocontrol and insecticides over the years, insecticide resistance amongst vectors is still a problem. This is, therefore, increasingly impacting on the success of malaria control interventions (reviewed by Coetzee *et al.,* 1999; Ranson *et al.,* 2009). Insecticide resistance has been reported in many malaria vectors worldwide and the understanding of the vector biology, resistance mechanisms and the insecticides in question will greatly attribute to the control of malaria.

1.4 Insecticide Resistance

Resistance is defined by the World Health Organization (WHO) as, "the inherited ability of a strain of some organism to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species" (WHO, 1957). Insecticide resistance can develop through mainly one or more of four mechanisms and these occur when (WHO, 1957; Hemingway *et al.,* 2004):

i) Resistant mosquitoes destroy or naturally detoxify the insecticide faster than the susceptible ones (metabolic resistance);
ii) The site at which the insecticide binds in the mosquito has been genetically altered to reduce its effects (altered target-site resistance);
iii) The resistant mosquitoes develop thicker cuticles thus reducing the rate at which the insecticide is being absorbed (penetration resistance) or
iv) Resistant mosquitoes may recognize and detect the threat and avoid the insecticide (behavioural resistance).

The bulk of insecticide resistance in mosquitoes to date is mainly through metabolic resistance and/or target site resistance. The other two mechanisms are more difficult to study and very little information is available on them.

1.4.1 Metabolic resistance mechanism/detoxification

The metabolic resistance mechanisms involve three major enzyme groups, carboxylesterases (esterases), P450 monooxygenases (P450s) and glutathione *S*-transferases (GSTs). These metabolic enzymes are responsible for the resistance to four classes of insecticides: organophosphates, organochlorines, carbamates and pyrethroids (Hemingway and Ranson, 2000) which are the only classes of insecticides approved for public health usage. Figure 1.2 shows the complex interaction between the insecticides, their target sites and metabolic enzymes involved. The monooxygenases and esterases are generally involved in organophosphate, carbamate and pyrethroid resistance. The GSTs are mainly involved in DDT resistance (Hemingway and Ranson, 2000).

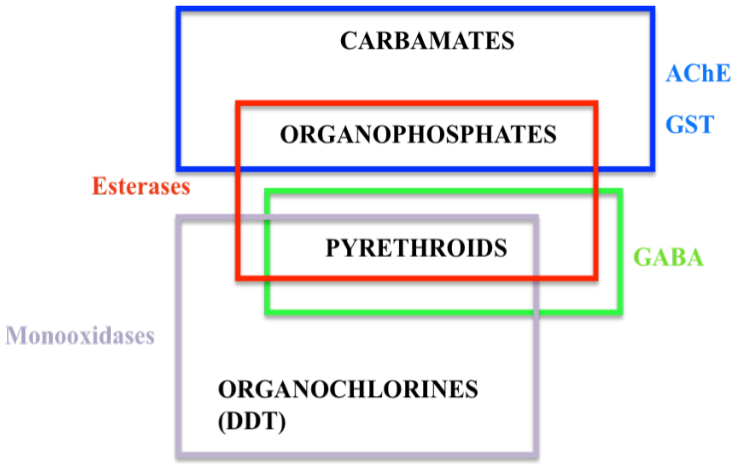

Figure 1.2 Diagrammatic representation of cross-resistance between commonly used classes of insecticides and the affected target site (adapted from Brogdon and McAllister, 1998)

1.4.1.1 Carboxylesterases

The carboxylesterase (esterase) resistance mechanism is involved in organophosphate (OP), carbamate and in some cases pyrethroid insecticide resistance (Hemingway and Karunaratne, 1998). Insect esterase genes are subdivided into eight subfamilies according to sequence similarity and substrate specificity and these include: α-esterase, ß-esterase, juvenile hormone esterase, gliotactins, acetylcholinesterases, neurotactins, neuroligins, and glutactin class (Ranson *et al.,* 2002a). The α-esterase, ß-esterase, juvenile hormone esterase and acetylcholinesterases account for most of the catalytically active esterases whilst the remaining esterases are non-catalytic and are involved in development and neurogenesis (Ranson *et al.,* 2002a; Yu *et al.,* 2009).

Esterases are known to play a role in the detoxification and metabolism of xenobiotic compounds and insecticides (Russell *et al.,* 1996; Li *et al.,* 2007), hydrolysis of the juvenile hormone, neurotransmitter acetylcholine as well as the hydrolysis of carboxylic esters (Taylor and Radic, 1994; Hemingway and Karunaratne, 1998; Riddiford *et al.,* 2003). Esterases have also been reported to play a role in organophosphate resistance in *Culex quinquefasciatus* from Saudi Arabia (Hemingway *et al.*, 1990).

In *An. gambiae* there are 51 known gene sequences of the esterase family (Ranson *et al.,* 2002a). To date there have been reports of esterases playing a role in cross-resistance in *An. funestus* using biochemical analysis (Brooke *et al.,* 2001) but there are no reports on gene transcription studies done on this enzyme family.

1.4.1.2 Cytochrome P450 (monooxygenases)

Cytochrome P450 or monooxygenases consists of a large, ancient super family of heme-thiolate proteins and are found in most insects, plants, mammals, birds and bacteria (Scott, 1999; Alzahrani, 2009). P450s are membrane-bound enzymes and are found mainly in the endoplasmic reticulum or in the mitochondrial membrane (Alzahrani, 2009). P450s are found in most cell tissues; however, in insects it has been found to be located mainly in the midgut, fat bodies and malpighian tubules (Hodgson, 1983). Most eukaryotic P450s require the flavoprotein NADPH, cytochrome P450 reductase and cytochrome b_5 for activity (Hemingway *et al.,* 2004).

Due to P450s comprising a large number of proteins, the naming of each one is imperative. Therefore, a standardized naming system was implemented (Nelson *et al.,* 1993). In the present system, cytochrome P450 genes are named *CYP* (**Cy**tochrome **P**450) followed by a number (family name), letter (subfamily) and number (isoform) for example, *CYP6P9* (Nelson *et al.,* 1996; Feyereisen, 1999; Scott, 1999; Ranson *et al.,* 2002b). Members of a family share more than 40% identity at the amino acid sequence level and more than 55% at the subfamily level (Bergé *et al.,* 1998; Feyereisen, 1999; Werck-Reichhart and Feyereisen, 2000; Ranson *et al.,* 2002b).

Due to the large number of P450 gene families, approximately 4000 being discovered across living organisms, (Nelson, 2006), blocks of two to four digit numbers were assigned for different orders of organisms; CYP1-CYP49, CYP301-CYP499 and CYP3001-CYP4999 are reserved for animals, CYP71-CYP99, CYP701-CYP999 and CYP7001-CYP999 for plants, CYP51-CYP69, CYP501-CYP699 and CYP5001-CYP6999 belong to lower eukaryotes, CYP101-CYP299 and CYP1001-CYP2999 to bacteria (Nelson, 2006).

In insects, as of 2009, there were 1 675 reported P450s in six P450 families. These six families include CYP 6, 9, 12, 18 and 28 while the sixth family, CYP4, also includes sequences from vertebrates (Bergé *et al.,* 1998; Feyereisen, 1999; Ranson *et al.,* 2002a; Alzahrani, 2009). Some CYP4 enzymes are thought to play a role in resistance to xenobiotics, mobilization of energy and fatty acids oxidation (Alzahrani, 2009). The CYP6 and CYP28 families are also thought to play a role in metabolism of xenobiotic compounds. The CYP12 P450 family is mitochondrial and plays a role in steroidogenesis (Alzahrani, 2009). The CYP12 P450s have also been found at higher levels in insecticide resistant strains of insects (Le Goff *et al.,* 2006). The function of CYP9 has not clearly been established (Danielson *et al.,* 1997), while the CYP18 enzymes function in C26 hydroxylation/oxidation (Feyereisen, 2006).

In *An. gambiae* a total of 111, P450 genes (of which seven are pseudo genes) have been identified (Ranson *et al.,* 2002a). In 2005, Amenya *et al.* identified 31 P450 genes in *An. funestus*. In the Amenya study, a total of 12 CYP4, 12 CYP6 and seven CYP9 partial genes were isolated and sequenced.

P450 enzymes function by binding molecular oxygen and receiving electrons from NADPH (nicotinamide adenine dinucleotide phosphate). The enzyme in turn adds an oxygen atom to the substrate and forms water with the other oxygen atom (Bergé *et al.,* 1998). This process is represented in Figure 1.3. Electrons are transferred from NADPH on the substrate-P450 complex by a NADPH cytochrome P450 reductase. These reactions can also occur in the presence of cytochrome b_5 (Bergé *et al.,* 1998).

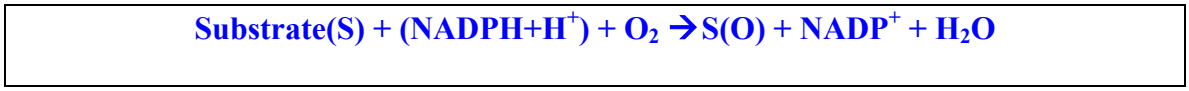

Figure 1.3 The metabolic reaction of P450 cytochromes

P450s are considered to be an important enzyme group, due to their involvement in the detoxification or activation of xenobiotics such as drugs or pesticides and the metabolism of compounds (Bergé *et al.,* 1998; Feyereisen, 1999; Scott, 1999; Brown *et al.,* 2003). Monooxygenases are known to oxidize a large number of substrates and catalyze a large range of reactions (Scott, 1999). They are also known for their involvement in controlling the production of compounds such as hormones, fatty acids, pheromones and steroids (Scott, 1999; Brown *et al.,* 2003).

Monooxygenases are interesting to study due to their large variation in substrate specificity (Scott, 1999). Some P450s display overlapping substrate specificity such that a single compound may be subject to metabolism by multiple P450s. In some cases, some P450s can produce only one metabolite from a given substrate, whilst other P450s produce several metabolites (Scott, 1999).

It is well known that elevated levels of P450s result in insecticide resistance in many insect species (Liu and Scott, 1998; Feyereisen, 1999; Kasai *et al.,* 2000; Ranson *et al.,* 2002a; David *et al.,* 2005; Djouaka *et al.,* 2008; Müller *et al.,* 2008b; Awolola *et al.,* 2009). Subfamilies from monooxyegenase have been reported to be involved in the metabolism of all insecticide classes and these include CYP4, CYP6 and CYP12 (Guzov *et al.,* 1998; Ranson *et al.,* 2002a; Nikou *et al.,* 2003; Pridgeon *et al.,* 2003; Rongnoparut *et al.,* 2003). A study conducted by Nikou *et al.* (2003) found the *CYP6Z1* gene to be overexpressed in a pyrethroid resistant *An. gambiae* strain. These enzymes have also been found to be up-

regulated in other insects such as the *CYP6L1* gene in adult male German cockroaches, *Blattella germanica (L)* (Wen and Scott, 2001), in *Drosophila melanogaster* (Chung *et al.,* 2009) and the *CYP6A36* and *CYP6A37* genes in the house fly, *Musca domestica* (Zhu *et al.,* 2008).

1.4.1.3 Glutathione *S*-transferases

Glutathione *S*-transferases are members of a large family of multifunctional intracellular enzymes and are found in most living organisms (Ding *et al.,* 2003; Che-Mendoza *et al.,* 2009). There are over 40 GST genes and three known groups of GST protein families in eukaryotes and these include the microsomals or kappa (κ) group (membrane-associated proteins), cytosolics (occurs in the cytoplasma) and the mitochondrials (found in the mammalian mitochondria and peroxisomes) (Che-Mendoza *et al.,* 2009).

GSTs that have over a 40% sequence identity, have phylogenetic relationships, share immunological properties, tertiary structures and the ability to form heterodimers, are assigned to the same class (Hemingway *et al.,* 2004). There are eight cystolic GST classes in mammals, Alpha (α), Mu (μ), Pi (π), Theta (θ), Sigma (Σ), Zeta (Z), Kappa (κ) and Omega (Ω) and a microsomal class (Ranson *et al.,* 2001). There are six known classes of GSTs in insects, Delta (δ), Epsilon (ε), Omega (Ω), Sigma (Σ), Theta (θ), Zeta (Z), an unknown class and a microsomal class. Delta and Epsilon are the largest classes found only in insects (Enayati *et al.,* 2005). GSTs are named according to the species they were isolated from and the GST class they belong to. They are also assigned a number that indicates the order in which they were discovered or genomic organization (Hemingway *et al.,* 2004). For example, in the gene *AgGSTe4,* Ag refers to the species in which it was

discovered, *An. gambiae,* and e4 indicates that it belongs to the Epsilon class and was the fourth GST in that class to be identified (Enayati *et al.,* 2005).

In *An. gambiae* there are 31 known GSTs (Ranson *et al.,* 2002a). Twelve of these genes belong to the Delta class, eight to Epsilon, one to Omega, one to Sigma, two to Theta, one to Zeta and three to both the unknown and microsomal classes (Claudianos *et al.,* 2006). There have been reports on increased GST activity in *An. funestus* using biochemical analysis (Cuamba *et al.,* 2010; Morgan *et al.,* 2010) but no gene transcription studies have been conducted on GSTs in this species.

Insect GSTs are of importance due to their involvement in detoxification of a wide range of xenobiotic compounds such as insecticides and these include organophosphates, organochlorines and pyrethroids (Ortelli *et al.,* 2003; Che-Mendoza *et al.,* 2009). GSTs play a role in the transport of proteins and ligans and in signaling processes (Hemingway and Ranson, 2000; Ding *et al.,* 2003; Ortelli *et al.,* 2003). They have also been known to play a role in protection against oxidative stress (Ding *et al.,* 2003; Che-Mendoza *et al.,* 2009).

1.4.2 Target-site resistance

The second most widespread mechanism by which insects are able to resist insecticides is through target-site resistance. Target-site insensitivity results when point mutations occur at the target sites of insecticides, decreasing the affinity of the insecticide to its receptor (Brogdon and McAllister, 1998). Alterations of the sodium ion channel gene knock-down resistance (*kdr*) which is the target site for DDT and pyrethroids, γ-aminobutyric acid (GABA) receptor which is the target of cyclodiene insecticides and acetylcholinesterase

(AChE) which is inhibited by organophosphate and carbamate compounds, result in the insecticide not being effective (Hemingway and Ranson, 2000; Djogbenou *et al.,* 2008). Details on these different target sites are provided in the following sections.

1.4.2.1 Insensitive acetylcholinesterase

Acetylcholinesterase (AChE) acts by hydrolyzing the excitory neurotransmitter, acetylcholine, on the post-synaptic nerve membrane (Hemingway and Ranson, 2000). Organophosphates and carbamates target the acetylcholinesterase and inhibit enzyme activity by carbamylating and phosphorylating the serine residue within the active site gorge (Hemingway *et al.,* 2004). There have been reports of two types of target-site resistance, i.e. one conferring high carbamate and low organophosphate resistance and the other, which has a high organophosphate with an equal or low carbamate resistance (Russel *et al.,* 2004). To date, all mosquito AChE target site resistance falls within the first category (Hemingway *et al.,* 2004). Studies conducted on *An. gambiae s.l* populations from Ivory Coast (N'Guessan *et al.,* 2003) and Burkina Faso (Djogbenou *et al.,* 2008; Dabiré *et al.,* 2009) have all shown the presence of the G119S mutation of the *ace-1*^{R} gene, which is responsible for insensitive acetylcholinesterase resistance to organophosphate and carbamate insecticides.

1.4.2.2 GABA receptor mutation

The GABA (γ-aminobutyric acid) receptor is a heteromultimeric gated chloride-ion channel and an inhibitory neurotransmission channel in an insect's central nervous system and in neuromuscular junctions (Hemingway and Ranson, 2000). It consists of five subunits, which are arranged at the central ion channel (Hemingway *et al.,* 2004). The GABA receptor is the site for cyclodiene insecticides such as dieldrin (Hemingway and

Ranson, 2000; Hemingway *et al.,* 2004). In most dieldrin resistant insects, a single mutation occurs from the alanine residue to a serine (Ffrench-Constant *et al.,* 1993). In 2005, Du *et al.* carried out one of the first studies which showed that resistance to dieldrin in *An. arabiensis* occurs when this same mutation is present. This mutation has been called *Rdl*. The same study also discovered that a different mutation of alanine to glycine occurred in *An. gambiae*.

1.4.2.3 Mutations in the voltage-gated sodium channel

This form of resistance occurs when there are mutations in the voltage-gated sodium channel and the insecticides, DDT and pyrethroids are prevented from being active at this site (Hemingway *et al.,* 2004). The intensive use of insecticides has led to the development of knock-down resistance (*kdr*) in Anopheles mosquitoes (Liu *et al.,* 2000). The knock-down resistance (*kdr)* mutation was first detected in houseflies and the German cockroach (Knipple *et al*., 1994; Liu *et al*., 2000). One type of resistance-associated mutation in *kdr* in *An. gambiae* and which is predominant in west Africa (*kdr*-w), results in a leucine-to-phenylalanine substitution (L1014F) in the S6 hydrophobic segment of domain II (Williamson *et al.,* 1996; Martinez-Torres *et al.,* 1998). This mutation is associated with *kdr* to pyrethroids and DDT. Two other different mutations at this position also confer resistance to DDT and/or pyrethroids. The first is a leucine–histidine substitution which is associated with pyrethroid resistance in *Heliothis virescens* (Park & Taylor, 1997). The second is a leucine–serine substitution (L1014S) which confers DDT resistance and low levels of permethrin resistance in *An. gambiae s.s* from east Africa (*kdr*-e) (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000). Another type of mutation, the methionine–threonine mutation, has been detected in the housefly showing high levels of pyrethroid resistance (Lee *et al.,* 1999).

1.4.3 Behavioural resistance

Behavioural resistance results when some insecticides cause insects to avoid or leave treated surfaces before acquiring a lethal dose of insecticide such that frequent contact is necessitated before death occurs (WHO, 1957). Behavioural resistance is divided into two categories, contact irritancy and non-contact repellency. Contact irritancy occurs when the insect is stimulated to leave an insecticide treated surface after physical contact. Non-contact repellency results when the insect is stimulated to leave an insecticide treated area before contact is made with the treated surface (Georghiou, 1972; Potikasikorn *et al.,* 2005; Liu *et al.,* 2006). This form of resistance has been reported for many classes of insecticides and these include, pyrethroids, carbamates, organochlorines and organophosphates.

Behavioural resistance is challenging to control and very little information is available regarding the management strategies and the mechanism. However, there have been a few studies investigating this form of resistance. The *An. minimus* complex species A and C both demonstrated irritancy and escaped from chambers after being exposed by contact and non-contact to DDT and pyrethroids (Potikasikorn *et al.,* 2005). Studies conducted on *An. albimanus* (Chareonviriyaphap *et al.,* 1997), *An. minimus* (Chareonviriyaphap *et al.,* 2001), *An. minimus* (Sungvornyothin *et al.,* 2001) and *Ae. aegypti* (Kongmee *et al.,* 2004) all showed forms of behavioural resistance.

1.4.4 Penetration resistance

Penetration resistance results when the insect's exoskeleton has been modified such that foreign compounds are prevented from entering its body. Reducing the infiltration of the insecticide through the insects adapted cuticle will result in the body having ample time to

detoxify the foreign substances, hence being less effective (Plapp, 1976). This results in the slow absorption of the insecticides into their bodies. This type of resistance usually occurs together with other forms of resistance (IRAC, 2009). Very few studies have investigated this type of resistance, particularly in mosquitoes. Studies conducted on cotton bollworm *Helicoverpa armigera* in Thailand (Ahmad and McCaffery, 1999) and west Africa (Martin *et al.,* 2002) found that this species is resistant to pyrethroids due to cuticular thickening. Decreased penetration of insecticide has been found in the housefly, *Musca domestica* (L.) (Wen and Scott, 1999). The molecular basis of this mechanism is yet to be investigated. Both Djouaka *et al.* (2008) and Awolola *et al.* (2009) have found overexpression of cuticular genes in pyrethroid resistant *An. gambiae.* Recently, Wood *et al.* (2010) showed that individual pyrethroid resistant *An. funestus* mosquitoes had a thicker cuticle than their susceptible counterparts. However, the role this plays in resistance is still unclear as the main resistance mechanism in this population is metabolic of nature (Brooke *et al.,* 2001).

1.5 Malaria in South Africa

The main malaria areas in South Africa include parts of the Limpopo province, Mpumalanga and KwaZulu Natal (http://www.doh.gov.za/). The vectors responsible for malaria transmission are mainly *An. arabiensis* and *An. funestus* when it is allowed to encroach into the sprayed areas. There have been reports of insecticide resistance in malaria vectors in southern Africa and these include DDT and pyrethroid resistance in *An. arabiensis* in South Africa (Hargreaves *et al.,* 2003; Mouatcho *et al.,* 2009) and *An. funestus* resistant to pyrethroids and carbamates in South Africa and Mozambique (Hargreaves *et al.,* 2000; Brooke *et al.,* 2001).

The first account of insecticide resistance in *An. funestus* in southern Africa and its impact on malaria transmission was reported by Hargreaves *et al.* (2000). During 1996, South Africa discontinued DDT in favour of deltamethrin (pyrethroids) for indoor residual house spraying, unaware that the neighbouring population of *An. funestus* in southern Mozambique had developed resistance to pyrethroids (Hargreaves *et al.,* 2000). As a result, South Africa experienced its worst malaria epidemic in over 50 years (Figure 1.4).

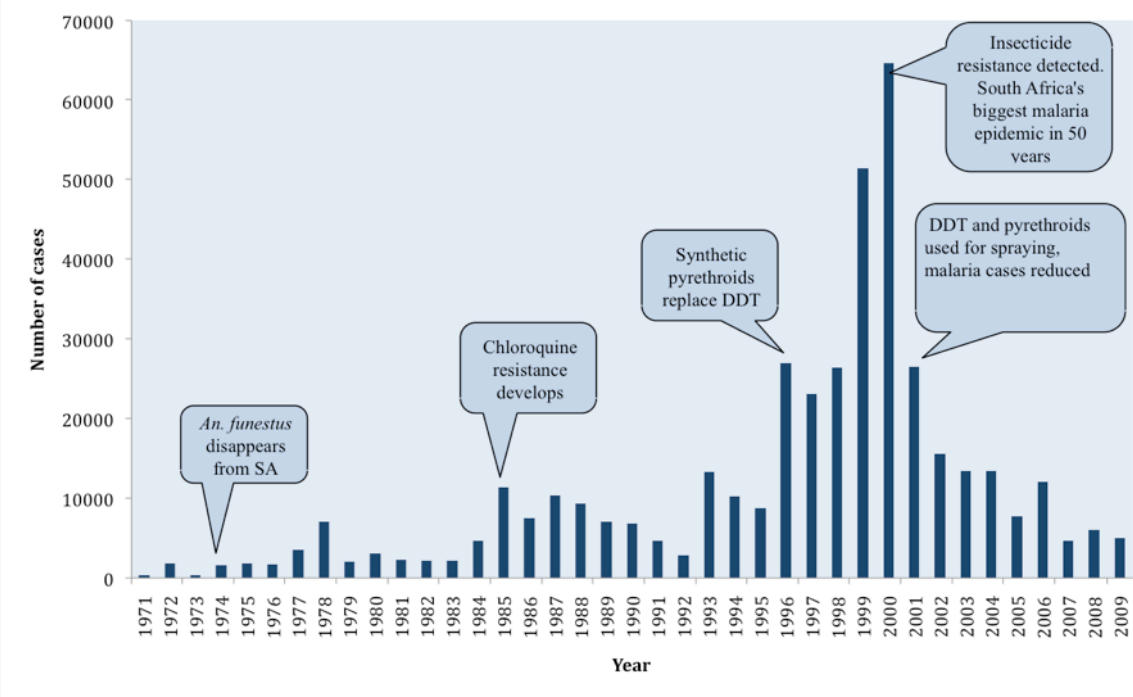

Figure 1.4 Malaria cases and deaths in South Africa from 1971-2009 (Department of Health, 2010, unpublished reports)

Malaria cases in South Africa peaked at 64 000 during the 1999/2000 malaria season (Coetzee, 2005). Effective control was re-established by using DDT in traditional houses, whilst retaining pyrethroids for use in western style houses. It was subsequently established that pyrethroid and carbamate resistance in *An. funestus* from South Africa and southern Mozambique was based on monooxygenase detoxification (Brooke *et al.,* 2001). The malaria situation in South Africa has since returned to pre 1996 levels. Presently, the continuous report of malaria infections in South Africa is due to travelling between South

Africa and neighbouring malarial countries such as Mozambique and Zimbabwe (Department of Health, 2010, unpublished reports).

1.6 Insecticide Resistance in South Africa

An understanding of resistance mechanisms in vector populations enables the development of resistance management strategies and ultimately the control of malaria. Hence, it is imperative to study the vector's phenotype in response to exposure to insecticides, as well as the resistance mechanisms involved at the molecular level. Very few molecular studies have been reported on *An. funestus.* Studies by Brooke *et al.* (2001) found that *An. funestus* from South Africa and southern Mozambique showed evidence of resistance to pyrethroid and carbamate insecticides. Results showed that high levels of monooxygenases were responsible for the detoxification of pyrethroids in the resistant mosquitoes and this was carried out using insecticide exposure, synergist and biochemical assays. Additional pyrethroid resistance studies were carried out by Hunt *et al.* (2005) showing that *An. funestus* resistant mosquitoes exhibited increased monooxygenase and glutathione *S*-transferase activity.

Up until this point no information was available on the genes involved in the elevated activity of monooxygenase and glutathione *S*-transferase. However, in 2005, Amenya *et al.* isolated 31 partial P450 CYP genes. These gene sequences had a 75% identity to *An. gambiae* amino acid sequence level. This information gave insight into the genes that may possibly be involved in insecticide resistance. In 2008, Amenya *et al.* conducted one of the first studies to use molecular techniques to identify a gene, *CYP6P9,* which was thought to play a role in insecticide resistance in a pyrethroid resistant strain of *An. funestus* from

Mozambique. Genetic mapping studies by Wondji *et al*. (2007) identified the P450 gene, *CYP6P9* as being highly overexpressed in the resistant FUMOZ-R strain. Two years later, Wondji *et al.* (2009) identified a duplicate gene of *CYP6P9,* called *CYP6P9b*. Furthermore, Matambo *et al.,* (2010) identified several single nucleotide polymorphisms (SNPs) between the full-length *CYP6P9* gene from the resistant and susceptible *An. funestus* strains. Another CYP gene, named *CYP6P13,* was found to be closely related to the *CYP6P9* gene. Amino acid comparisons between *CYP6P13* and *CYP6P9b* implied that these are the same gene (Wondji *et al.,* 2009; Matambo *et al.*, 2010).

1.7 Objectives

Very few studies have been carried out to characterize the complex resistance mechanisms in *An. funestus.* The research done thus far has confirmed pyrethroid resistance in *An. funestus* and suggested resistance to carbamates. Molecular research on *An. funestus* has not progressed at the same rate as *An. gambiae* due to the difficulty of rearing *An. funestus* in the laboratory.

This study aimed to examine the effects of pyrethroid resistance on transcription of metabolic enzymes in *An. funestus.* The objectives of this study are listed below and the workflow for each objective is presented in Figure 1.5.

1.7.1 Objective one

a) To investigate the levels of pyrethroid resistance at different ages in males and females of a resistant *An. funestus* strain.

b) The expression profiles of genes, *CYP6P9* and *CYP6P13*, that have been previously reported to play a role in pyrethroid resistance in *An. funestus,* were determined at these different ages.

1.7.2 Objective two

a) To determine the transcription profile of detoxification genes in both the susceptible and pyrethroid resistant strain of *An. funestus* using the *An. gambiae* detoxification chip (detox chip).

1.7.3 Objective three

a) To determine the transcription profile of the detoxification genes in the first instar, fourth instar and 3-day old adult mosquitoes using the *An. gambiae* detox chip.

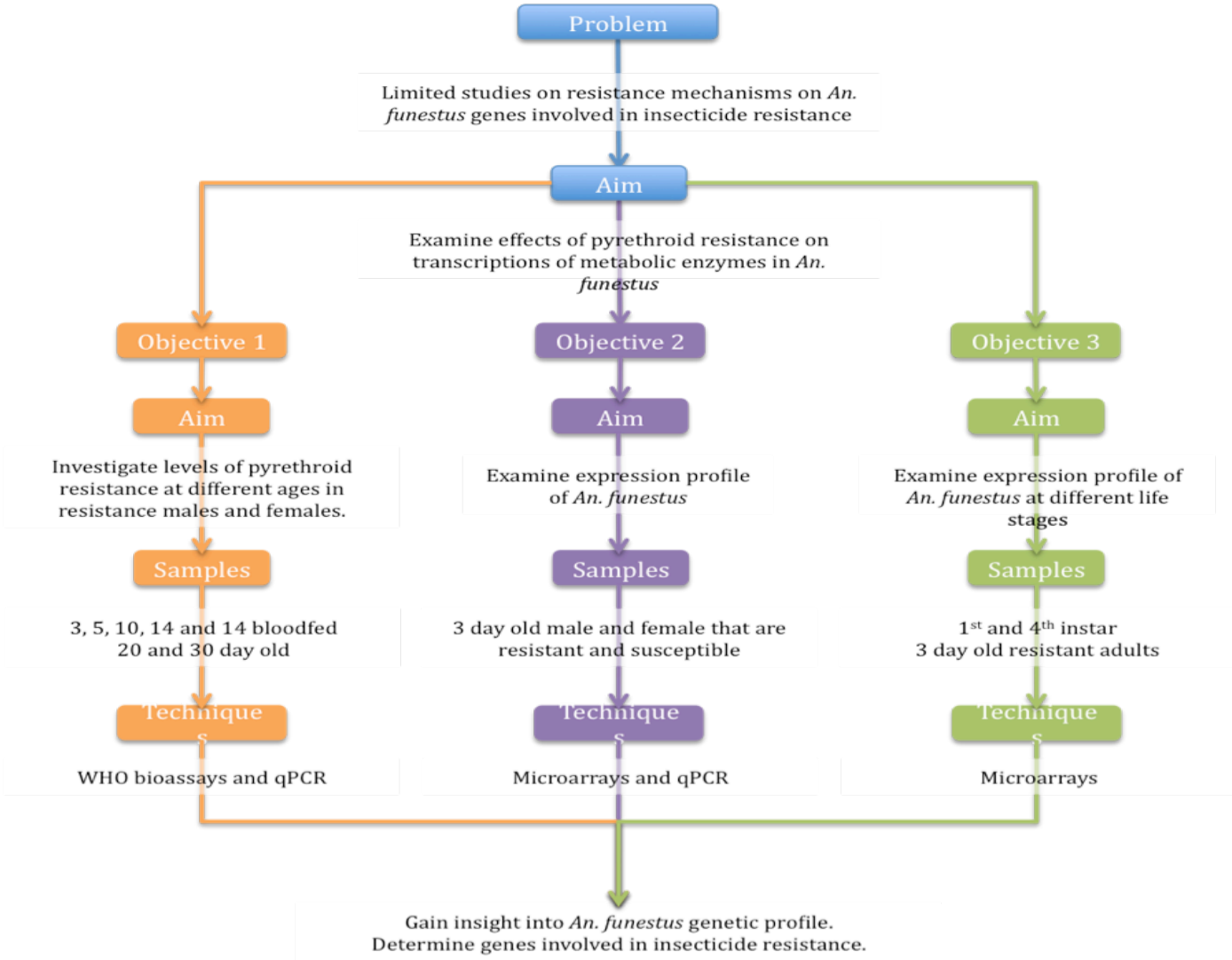

Figure 1.5 Workflow of the aims, materials and techniques used in this study

CHAPTER TWO

The effect of age on pyrethroid resistance and the expression level of two duplicate P450 genes in *Anopheles funestus*[*]

2.1 Background

Various insecticide resistance mechanisms have been described and molecular techniques have been used successfully to provide a better understanding of these mechanisms (Nikou *et al.,* 2003; Vontas *et al*., 2007; Wondji *et al.,* 2007; Amenya *et al.,* 2008; Ben Cheikh *et al.,* 2009). Insecticide resistance in *An. gambiae* and *An. funestus* has previously been described (Hargreaves *et al*., 2000; Hemingway and Ranson, 2000; Coetzee and Fontenille, 2004; Munhenga *et al.,* 2008; Okoye *et al.,* 2008; Awolola *et al.,* 2009). However, the molecular understanding of these mechanisms in *An. gambiae* far out-weighs that of *An. funestus.* The availability of the *An. gambiae* genome has enlarged the gap in information between these two species. It is only recently that the molecular mechanism of monooxygenase-based pyrethroid resistance in *An. funestus* was initiated despite the lack of available genomic sequence data (Amenya *et al.,* 2005; Wondji *et al.*, 2007; Amenya *et al.,* 2008; Matambo *et al.,* 2010). Recently, Serazin *et al.* (2009) reported the functional annotation and comparative genomics of 2 005 expressed sequenced tags from *An. funestus* – information which will prove useful in future molecular characterisation studies. The information from this study also adds to the body of molecular information available for *An. funestus.*

* Footnote: Accepted for publication in Journal of Genetics and Molecular Research. See Appendix C, page 148.

Initial molecular characterisation of pyrethroid resistance in *An. funestus* included the isolation of 31 partial P450 sequences (Amenya *et al.,* 2005). One of these, *CYP6P9*, was shown to be overexpressed in the egg and adult stages of a pyrethroid resistant strain from Mozambique (Amenya *et al.,* 2008). Wondji *et al.* (2007) identified a quantitative trait locus (QTL) associated with pyrethroid resistance in the same *An. funestus* strain. These QTL markers contained a cluster of P450 genes including the *CYP6P9* gene. Recently, Wondji *et al.* (2009) have also identified a duplicate gene of *CYP6P9,* and called this gene, *CYP6P9b*.

Full-length *CYP6P9* sequence comparisons between the resistant and susceptible *An. funestus* strains identified several single nucleotide polymorphisms (SNPs) (Matambo *et al.* 2010). Furthermore, an additional CYP6, closely related to *CYP6P9*, was identified and named by the Dr Nelson (http://drnelson.utmem.edu/CytochromeP450.html) P450 Nomenclature committee as *CYP6P13* (Matambo, 2008). Based on amino acid similarities it seems likely that *CYP6P13* and *CYP6P9b* are the same gene (Wondji *et al.,* 2009; Matambo *et al.*, 2010).

This newly obtained information from the latter two publications resulted in confusion as to the role of *CYP6P9* expression in pyrethroid resistance. Expression studies conducted by Amenya *et al.* (2008) as well as by Wondji *et al.* (2009) were aimed at "*CYP6P9*" without differentiation between *CYP6P9* and *CYP6P9b*/*CYP6P13*. The study in this chapter aimed to characterize the role of these genes separately in pyrethroid resistance. Due to the confusion in naming and differentiating between these two genes the following nomenclature was used:

i. Combined effect of *CYP6P9* and *CYP6P13*: this naming was used to imply that amplicons produced are similar to amplicons from previous literature by Amenya *et al.* (2008) and Wondji *et al.* (2009). Amplicons were obtained using the same primers as described by Amenya *et al.* (2008).

ii. *CYP6P9*: this implies that the amplicon produced is specific for *CYP6P9* (Matambo *et al.*, 2010) or *CYP6P9a* named by Wondji *et al.* (2009). As Wondji *et al.* (2009) did not name either *CYP6P9a* or *b* it was decided to use the formal naming system as obtained by the Dr Nelson (http://drnelson.utmem.edu/CytochromeP450.html) P450 Nomenclature committee. New primer pairs were designed specific for this gene.

iii. *CYP6P13*: this implies that the amplicon produced is specific for *CYP6P13* or *CYP6P9b* (Wondji *et al.,* 2009). The same reason for naming was used as described above. New primer pairs were designed specifically for this gene.

In order to study the expression of these genes, the mRNA of each had to be determined. Determining the mRNA levels of a gene can be done using quantitative Real-Time PCR or qPCR. qPCR is one of the most sensitive methods for the detection of the amount of DNA/cDNA in a sample (Stahlberg *et al.,* 2005). In conventional PCR, the amplified product is only detected at the end of the PCR reaction on an agarose gel (end point analysis). In qPCR however, the amount of amplified PCR product and detection of the product is determined at every step as the reaction progresses in "real time" (Bio-Rad, 2006). Sufficient product accumulates to yield a fluorescent signal at a certain point. The cycle number at which this occurs is called the quantification cycle, C_Q value. Higher copy number of target DNA or RNA in the starting material will result in a significant increase in fluorescent signal, which in return will result in a lower C_Q value. The detection of the

PCR product is achieved by fluorescent molecules (SYBR Green) that bind to the double stranded (ds) DNA. The fluorescent signal in a reaction is therefore relative to the amount of (ds) DNA present and will increase as the target is amplified (Bio-Rad, 2006). The general steps followed in a qPCR reaction are outlined in Figure 2.1.

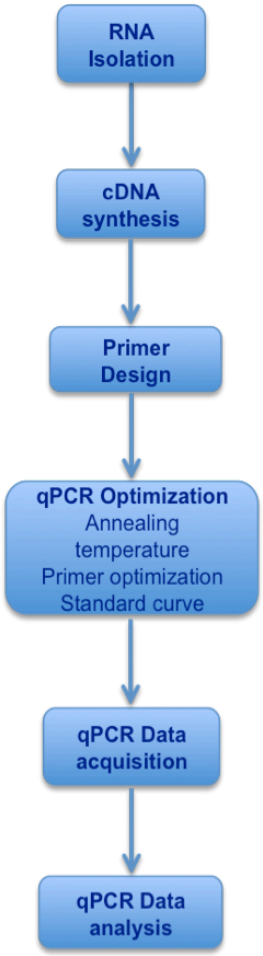

Figure 2.1 Flow diagram of steps involved in a qPCR reaction

There are two ways in which qPCR data can be analysed, i.e. absolute quantification or relative quantification. In absolute quantification, the C_Q value of a test sample is compared to a standard curve. The quantity of an unknown gene is interpolated using a range of standards of known quantity (Bio-Rad, 2006). The results are analysed by determining the copy number of a gene in a given amount of sample. In the relative

quantification method, the sample gene expression is determined by calculating the ratio or relative amount of the test (sample of interest) sample DNA to the calibrator (also known as the control) sample DNA.

2.2 Aim and Objectives

It is well known that the age of female mosquitoes contributes to the vectorial capacity of malaria-transmitting *Anopheles.* Once a female is infected with the *Plasmodium* parasite, there is generally an incubation period during which the parasite matures and is ready to be transmitted to a host. This usually occurs between 10-14 days of age (Cook and Sinkins, 2010). During this time mortality of females is high and only a small number of females go on to transmit malaria. The examination of mosquitoes' susceptibility to insecticide at different life stages greatly contributes to the understanding of vectorial capacity. Also, understanding if gene expression is affected by age and blood feeding greatly contributes in the control of these vectors.

The aim of these experiments was to quantify the effect of adult mosquito age on the expression of pyrethroid resistance in *An. funestus* as well as to evaluate the mRNA expression of two P450 genes (*CYP6P9* and *CYP6P13*) in association with age.

2.2.1 Objective one

This objective was designed to quantify the levels of pyrethroid resistance at a range of ages of adult pyrethroid resistant *An. funestus* (3, 5, 10, 14, 20 and 30-days post emergence) using standard WHO insecticide susceptibility bioassays.

As blood feeding female mosquitoes are potentially infectious and able to transmit malaria from 10-days and older it is important to understand the impact of mosquito age on insecticide resistance. The results from this should indicate if the effectiveness of pyrethroids is age-related. In turn, this should also indicate the age at which adult *An. funestus* mosquitoes are most vulnerable when exposed to insecticides.

2.2.2 Objective two

This objective was designed to quantify the differential mRNA levels of *CYP6P9, CYP6P13* and the combined effect of both genes in pyrethroid resistant *An. funestus* in association with age.

2.3 Biological Material

The following *An. funestus* laboratory colonies were used in this study: FUMOZ-R, originating from southern Mozambique and selected for resistance to permethrin (pyrethroid), since 2001 and selected for approximately 45 generations. Selection for permethrin resistance has been halted in the last 5 years, however recent WHO bioassay tests have shown that FUMOZ –R has a 15 % mortality to 0.75% permethrin. Details on the colony, rearing, selection and insectary conditions can be found in Hunt *et al.* (2005). FANG, originating from southern Angola is fully susceptible to all insecticides. Female and male adult mosquitoes were separated on emergence and were maintained on a 10% sugar solution until they reached the ages of 3, 5, 10, 14, 20 or 30-days. Another group of 14-day old FUMOZ-R adult females were allowed to mate and take three blood meals. These females were given a blood meal when they were 6, 8 and 10-days of age. On day 14 they were used either for insecticide tolerance testing or for RNA extractions.

2.4 Methods

2.4.1 WHO insecticide bioassays

The insecticide susceptibility assays described in this study were carried out according to WHO specifications using WHO insecticide treated papers (WHO, 1998) (Figure 2.2). The efficacy of all test papers was first confirmed using samples of the susceptible strain, FANG. A 100% mortality was obtained when FANG was used.

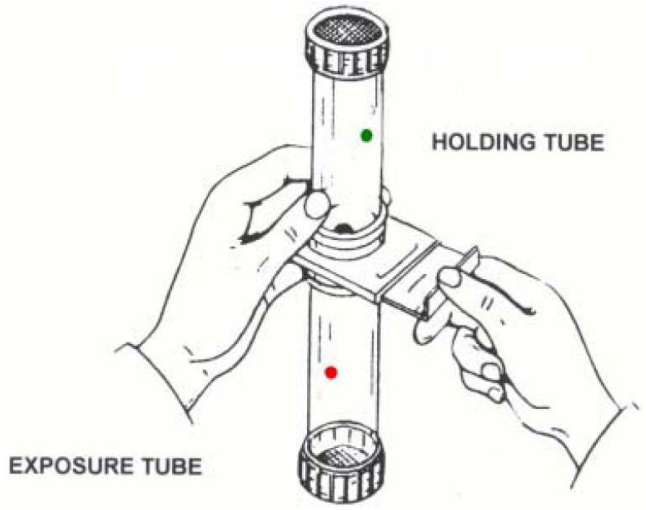

Figure 2.2 WHO standard insecticide testing tubes
Mosquitoes are placed into the holding tube (green dot) which is lined with untreated paper. The adults are then transferred to the exposure tube (red dot) lined with 0.75% permethrin for one hour. The adults are transferred back to the holding tube, the knockdowns recorded, and the mosquitoes fed 10% sucrose. The total number of dead and alive mosquitoes are recorded 24-hours post exposure (WHO, 1998)

A total of ± 30 FUMOZ-R adults per replicate were assayed against 0.75% permethrin treated papers. Ten replicates (in some age groups, more) for each age and sex cohort were used (total of 300 mosquitoes per age group). For each replicate an untreated control was included. Mortality in this control was never observed during this study. Knockdown was recorded after one-hour exposure and final mortality was recorded 24-hour post exposure.

Data analysis was conducted on final mortality rates as this is used to determine resistance (WHO, 1998). *Statistix 7* package (USA) was used to perform the analysis. The two sample *t*-test was used to compare the results of two different ages and linear regression was carried out to determine any relationship between all samples. Standard errors (SE) were calculated for each replicate.

Bioassay data were compared with expression levels of mRNA of the two genes, *CYP6P9*, *CYP6P13* and the combined effect of both these genes at different ages in order to ascertain any relationship between gene expression and pyrethroid resistance phenotype over age.

2.4.2 Quantitative Real-Time PCR (qPCR)

qPCR was conducted to:

i) Quantify FUMOZ-R mRNA expression of *CYP6P9*, *CYP6P13* and their combined effect for each age cohort.
ii) Determine the relative fold expression of *CYP6P9* and *CYP6P13* as a baseline quantification in 3-day old FANG samples.

An analysis of the combined effect was included for two reasons. Firstly, to allow for comparison with previously published work prior to the differentiation of these two genes (Amenya *et al.,* 2008; Wondji *et al.,* 2009). Gene expression studies by Amenya *et al.* (2008) and Wondji *et al.* (2009) showed that *CYP6P9* (defined in this thesis as the combined effect of *CYP6P9* and *CYP6P13*) is highly overexpressed in 3-day old pyrethroid resistant *An. funestus*. Analyses conducted on 14-day old adults showed an even greater level of expression than that of 3-day old adults (Amenya *et al.*, 2008). Therefore, qPCR, using three different primer pairs i.e. *CYP6P9, CYP6P13* and the combined effect

of both these genes at each of the different ages, was conducted to determine the mRNA levels of these genes in the resistant *An. funestus* colony.

Secondly, we hypothesise that the combined mRNA levels of *CYP6P9* and *CYP6P13* will be equal to the sum of the mRNA levels of *CYP6P9* and *CYP6P13* when analysed separately. If this hypothesis is not supported, it becomes conceivable that other P450 genes might also be involved in pyrethroid resistance in FUMOZ-R.

2.4.2.1 RNA isolation

RNA was extracted to prepare cDNA for the amplification of the *CYP6P9*, *CYP6P13* and the combined effect of both genes in qPCR. Total RNA was extracted from 15 adult *An. funestus* FUMOZ-R mosquitoes to form one biological repeat. Extractions were done separately for each sex and age cohort for FUMOZ-R as well as from 3-day old females and males from FANG. Three biological extractions were performed for each age and sex. The extractions were carried out using 600µl Tri reagent (Sigma, U.S.A, T9424). The homogenate was left to stand for five minutes at room temperature and 120µl of chloroform was added. The mixture was mixed and left to stand for a further five minutes. The homogenate was centrifuged at 12 000 rpm for 15 minutes at 4ºC. Three layers formed, and the top clear aqueous phase was collected and transferred into a clean 1.5ml microcentrifuge tube. Isopropanol (300µl) was added to the aqueous phase and left at room temperature for five minutes after which the sample was centrifuged at 12 000 rpm for ten minutes at 4ºC. The supernatant was removed and the RNA pellet was washed in 600µl of 70% ethanol. The pellet and ethanol mixture was centrifuged at 7 500 rpm for five minutes at 4ºC. The RNA pellet was air dried for five minutes and re-suspended in 30µl ddH_2O or DEPC water.

The RNA samples were DNase treated using the RNase-Free DNase set (Qiagen, Germany, 79254) by adding 35µl Buffer RDD and 5µl DNase I Stock Solution. The samples were incubated at room temperature for 15 minutes and quantified using a Nanodrop ® Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA was considered good quality if the samples had an A260/A280 ratio value between 1.8-2.0 and the concentration of the RNA was higher than 200ng/µl. The RNA was stored at -70ºC until further use.

2.4.2.2 cDNA synthesis

The cDNA was prepared for use in the qPCR component of this study. This was achieved by converting total RNA to cDNA. A solution of 1µl Oligo $(dT)_{20}$ (50µM) (Invitrogen, 18418020, USA), 1µl dNTP mix (10mM) and 1µl distilled water was added to 10µl of RNA (10pg-5µg). This mixture was incubated at 65ºC for five minutes and cooled on ice for one minute. To this RNA mixture, 4µl of 5X first strand buffer (Invitrogen, 18080044 USA), 1µl of dTT (0.1M) (Invitrogen, 18080044, USA), 1µl of RnaseOUT Recombinant Ribonuclease Inhibitor (40U/µl) (Invitrogen, 10777019, USA) and 2µl of Superscript III (200U/µl) (Invitrogen, 18080044, USA) was added. This was incubated at 50ºC for one hour and at 70ºC for 15 minutes. 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.

2.4.2.3 Primer design

Three sets of primers were used in this study: *CYP6P9, CYP6P13* and the combined effect of both these genes. Reasons for using these different primer sets are detailed below.

Matambo *et al.* (2010) have previously sequenced the *CYP6P9* and *CYP6P13* genes from the susceptible *An. funestus* strain, FANG, and carried out a sequence alignment between the two genes (Figure 2.3). The *CYP6P9* and the *CYP6P13* gene sequences showed 32 amino acid differences between them. When the forward primer sequence of the *CYP6P9* gene from the Amenya *et al.* (2008) study was located on the alignment between *CYP6P9* and *CYP6P13* (Figure 2.3), it was not located in the sequence-specific regions. This primer pair was designed in a region that contained no nucleotide differences between the two genes and it was able to amplify both *CYP6P9* and *CYP6P13* as indicated in the yellow region in Figure 2.3 (Matambo *et al.*, 2010). As there are no sequence differences between these genes in this location, one product is observed in the melt curve analysis as well as after sequencing. For this reason, we have called the amplicons obtained from the primer set designed by Amenya *et al.* (2008), "combined *CYP6P9* and *CYP6P13*". The forward and reverse primers of *CYP6P9*, *CYP6P13* and the combined genes are represented in Figure 2.3.



Figure 2.3 Nucleotide sequence alignment of *CYP6P9* and *CYP6P13* using FANG genomic DNA Nucleotide base differences are noted in grey shade and identical nucleotides are indicated with asterisks while similar residues are indicated with a dot (Matambo *et al.,* 2010). The green and yellow highlighted areas indicates the *CYP6P9* and "combined *CYP6P9* and *CYP6P13*" forward and reverse primers respectively, whilst *CYP6P13* forward and reverse primers are represented in the boxed areas.

Two additional primer sets were used in this study, *CYP6P9* (gene-specific primers) and *CYP6P13* (gene-specific primers). These two primer pairs, *CYP6P9* (GeneBank accession number: EU450763) and *CYP6P13* (GeneBank accession number: EF152577) were designed in the regions containing nucleotide differences, as illustrated in Figure 2.3. The *CYP6P9* primer was designed in this study as indicated in the green highlighted area, using the FUMOZ-R DNA sequence (in the same region as that illustrated in the FANG sequence) (Figure 2.3) whilst the *CYP6P13* primer was designed in the study conducted by Matambo (2008). Matambo (2008) designed two sets of primers for the *CYP6P13* gene and these include primer set (1) and primer set (2) (Table 2.1). A third primer set was generated and this included the *CYP6P13* forward primer from set (2) and the reverse primer from set (1). The *rsp 7* (EF450776) primers were used as a reference gene against which to normalise data.

These three *CYP6P13* primer sets and conditions outlined in Matambo *et al.* (2008) were used in a PCR amplification reaction. These primers were used to amplify both FANG and FUMOZ-R cDNA and genomic DNA in females. The amplicons of all three primer pairs were electrophoresed on a 1% agarose gel. The *CYP6P13* primer set which produced the best result was carried forward to the qPCR experiments. The cycling conditions outlined in the Amenya *et al.* (2008) study were carried out for the "combined *CYP6P9* and *CYP6P13*", whilst cycling conditions established in this study were used for the *CYP6P9* gene.

Table 2.1 Primers used in qPCR

Gene	Primer Sequence	Fragment Length (bp)	qPCR Annealing/Detection Temp (°C)	References
Combined effect (*CYP6P9 and CYP6P13*)	**Fwd** 5' GAG GAA GTG AAG AAG CGA CAT C 3' **Rev** 5' TGA CGG TGA GAA GCG GAA C 3'	140	52/ 84.00	Amenya *et al.,* 2008
CYP6P9	**Fwd** 5' AGA TGT GAT TGG CAC CTG T 3' **Rev** 5' TCG ATA TTC CAC CGT TTC CT 3'	232	55/ 82.00	Primer set designed in this study
CYP6P13 Primer set (1)	**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	Matambo, 2008
Primer set (2)	**Fwd** 5' ACT ATC AGG ACA CTT G 3' **Rev** 5' GAA AAA CTT CAT CAT AAT TAG GAG ATCC AG 3'			
Primer set (3)	**Fwd** 5' ACT ATC AGG ACA CTT G 3' **Rev** 5' GTT CAC CGT CTC GCG GAC T 3'			
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

* Annealing temperature for the *rsp 7* gene was 52ºC for the combined effect gene, 55ºC for *CYP6P9* and 59.1ºC for *CYP6P13*.

2.4.2.4 Quantification of *CYP6P9, CYP6P13* and the "combined effect of *CYP6P9* and *CYP6P13"*

The qPCR reactions were carried out 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, USA, 1708882), 4µl primer (0.4µM), 1µl cDNA (50ng) was used per reaction (Table 2.2). A standard curve was generated using a 2-fold serial dilution of cDNA for each of the target genes of interest and the reference gene. Serial dilutions of cDNA for the standard curve were prepared (Paton *et al.,* 2000; Bio-Rad, 2006). This was correlated to the initial cDNA template used in the reaction and then converted to transcript copy number (Bio-Rad, 2006).

Table 2.2 qPCR reagents and volumes used for combined genes primer set, *CYP6P9* and *CYP6P13*

Reagents	**Volume (µl)**	**Final Concentration**
SYBR Green mix	12.5	
Forward primer (2.5µM)	4	0.4µM
Reverse primer (2.5µM)	4	0.4µM
cDNA (50-150ng)	Variable	Variable
Distilled water	Variable	Variable
Total volume	**25**	

The cycling conditions for each primer set differed from each other. These conditions are presented in Table 2.3. Triplicates of each biological sample were carried out in each plate and the qPCR for the three different biological extractions was performed on different days. The reference gene, *rsp 7*, did not vary between the different ages when amplified. The identity of all amplified products was confirmed by sequence analysis by sequencing

in both directions. The sequenced genes were blasted against the *CYP6P9* and *CYP6P13* genes sequences to ensure that the correct product was amplified during qPCR.

Table 2.3 qPCR cycling conditions used for the *CYP6P9*, *CYP6P13* and the combined gene primer set, *CYP6P9* and *CYP6P13*

Genes	**Stage**	**Cycles**	**Temperature**	**Time**
Combined genes *CYP6P9* + *CYP6P13*	1	1	95ºC	3 mins
	2	35	95ºC	30 secs
			55ºC	30 secs
			72ºC	30 secs
	3	1	7 ºC	4 mins
CYP6P9	1	1	95ºC	3 mins
	2	39	95ºC	10 secs
			55ºC	15 secs
			72ºC	15 secs
	3	1	72ºC	30 secs
CYP6P13	1	1	94ºC	2 mins
	2	35	94ºC	30 secs
			59.1ºC	30 secs
			72ºC	40 secs
	3	1	72ºC	5 mins

2.4.2.5 qPCR data analysis

In order to proceed with qPCR data analysis a number of criteria needed to be met in the initial stages of the experiment. A valid qPCR experiment must have a PCR efficiency

percentage value between 90–105% for each gene. The R^2 (square of the coefficient of regression indicates how well the line fits the data) value must be as close to one as possible and the slope of the line must be greater than -3.00.

Two methods of data analysis were used in this study, the absolute quantification and the relative quantification method.

a) Absolute quantification

The qPCR data for the comparison of ages between 3, 5, 10, 14 unfed, 14 blood-fed, 20 and 30-day old adults were analysed using the absolute quantification method, as shown in the formula in Figure 2.4 (Bio-Rad, 2006). The values were converted into transcript copy numbers and the ratio of each gene to the reference gene, *rsp 7*, expression was determined (Bio-Rad, 2006).

$$N_n = 10\ (n-b)/\ m,$$

where N_n = copy number, n = C_Q, b = y intercept,

m = slope of line

Figure 2.4 Formula used to calculate the copy number of each gene

b) Relative quantification

Comparisons between 3-day old resistant versus susceptible mosquitoes were done using the relative quantification method (Figure 2.5) (Pfaffl, 2001).

$[(Etarget)\Delta C_Q, target(calibrator - test] \div [(Eref)\Delta C_Q, ref(calibrator- test)]$

Where target is the gene of interest, reference is the house-keeping gene,

test is the resistant sample and calibrator is the susceptible sample.

Figure 2.5 Formula used to calculate the relative expression of each gene

2.4.2.6 Verification of qPCR product

The amplified PCR products from the qPCR experiments were sent to Macrogen (Korea) for sequence verification of the synthesized product. Sequence analysis was conducted as described in section 2.4.2.7. It was expected that some PCR products that were < 140bp would be too small in size for sequencing to be carried out. The fragments that failed the first attempt of sequencing were then cloned into a vector to increase the size of the product, enabling subsequent successful sequencing.

a) Cloning

The PCR products from the qPCR reactions (25µl) were cleaned using the QIAquick Spin Kit (Qiagen, Germany, 28106). Five volumes of the PB buffer were added to one volume of the PCR sample. This sample mixture was applied to the QIAquick column and centrifuged for 30–60 seconds. The flow-through was discarded and 0.75ml of the PE buffer was added to the QIAquick column and centrifuged for 30–60 seconds. The flow-through was discarded and re-centrifuged for an additional minute. The QIAquick column was placed in a clean 1.5ml microcentrifuge tube and 50µl Buffer EB (10mM Tris·Cl, pH 8.5) or water was added to the column to elute the DNA. The column was centrifuged for one minute. The cleaned samples were nanodropped to determine the concentration of the purified samples.

b) Ligation

The ligation was performed using the pGEM-T Easy Vector kit (Promega, USA, A1360). The cDNAs from each of the genes were ligated into the pGEM-T Easy Vector and incubated at 4ºC overnight (Table 2.4). A positive and a negative ligation were also included. A positive ligation control is a reaction that contains a closed plasmid that is known to produce positive white clones. A negative control is one that contains all the reagents required in a ligation reaction without the PCR product (no closed plasmid will be present). This control should not produce any colonies in the transformation reaction due to no PCR product being present. If blue or white colonies are found in the negative, this could indicate that contamination had occurred during the ligation process and the results obtained in the sample reaction could be a false positive. The ligation process then has to be repeated.

Table 2.4 Ligation reaction for the cloning of the CYP and *rsp 7* genes into pGEM-T Easy Vector

Reagents	**Volume (µl)**
2x Rapid Ligation Buffer, T4 DNA Ligase	5
pGEM-T Easy Vector (50ng)	1
PCR Product (50ng/µl)	3
T4 DNA Ligase (3 weiss unit/unit)	1
Total volume	**10**

c) Transformation

The ligation reactions were removed from the fridge (4ºC) and incubated at 72ºC for 15 minutes. Approximately 5µl of the ligation reaction was added to 50µl *E. coli* JM109 competent cells, which were thawed on ice. A positive transformation was also carried out and this control is included in the transformation kit. The reaction was mixed gently by

flicking the tube and stored on ice for 20 minutes. The transformation reactions were heat-shocked at 42ºC in a water bath for 45 seconds and immediately stored on ice for two minutes. Luria Bertani (LB) broth ("LB"- see Appendix A for recipe) (960µl) or recovery medium from the *E. coli* JM109 kit was added to the cells and incubated at 37ºC for two hours with shaking at 200 rpm. LB plates containing ampicillin (AMP) (100µg/ml) were made (see Appendix A for recipe) and 16µl IPTG (50mg/ml) and 40µl of XGAL (20mg/ml) were added to plates once they were cooled. The transformation mixture (100µl) was added to the agar plates and spread using a sterile spreader. The plates were incubated at 37ºC overnight.

d) Colony screening

On day three, the plates were kept in the fridge (4ºC) for ± two hours to bring out the white colouration. Positive white clones were those that had the insert and sample of interest. The blue colonies contained no insert. The positive white clones of interest were selected and screened in a PCR reaction to identify the correct clone fragments (Table 2.5). The PCR cycling conditions are presented in Table 2.6. Ten clones per gene were selected from a plate for PCR screening. Five positive ligation samples were also selected and added for PCR. The selected remaining colonies of interest were added to 1-2ml of LB/AMP (50µg/ml) and were left to grow overnight at 37°C. The samples were added to 30% glycerol and stored at -70°C.

Table 2.5 PCR reaction of the cloned CYP and *rsp7* genes

Reagents	**Volume (µl)**
10 x PCR buffer	2.5
$MgCl_2$ (25mM)	1.5
dNTPs	2.0
*SP6 primer 6 pmol	0.6
*T7 primer 6 pmol	0.6
Sample	Variable
dH_2O	17.55
Taq (5U)	0.25
Total volume	**25**

Table 2.6 Cycling conditions used in the PCR reaction of the cloned products, CYP and *rsp 7* genes

Stage	**Cycles**	**Temperature**	**Time**
1	1	94ºC	3 mins
2	25	94ºC	30 secs
		56.5ºC	30 secs
		72ºC	30 secs
3	1	72ºC	10 mins

The PCR products were electrophoresed on a 1-2% gel and 3-5µl of the sample was loaded on an agarose gel. The samples were electrophoresed at 100 volts for 90 minutes. Positive PCR products that were correctly identified from their fragment size on the gel were selected and sent to Macrogen (Korea) for sequencing.

2.4.2.7 Sequence analysis

The sequences for all the products were obtained on the Macrogen website (http://dna.macrogen.com). The sequence data were aligned against the available sequences on BLAST (http://blast.ncbi.nlm.nih.gov). The percentage similarity was determined and if the gene of interest had a percentage value greater than 98% to the deposited sequences on the NCBI website then it was taken that the correct gene was used in qPCR and sequenced. Alternatively, the sequence obtained from Macrogen and the full/partial length sequences of the genes of interest were copied into the program DNASTAR Lasergene EditSeq (2007) and checked manually. These gene sequences were saved and then loaded to the DNASTAR Lasergene Megalign (2007) package. The sequences were aligned to each other using the Jotun Hein Method. The percentage similarities were then determined.

2.5 Results

2.5.1 WHO insecticide bioassays

Percentage survival of pyrethroid resistant adults (FUMOZ-R) 24-hours post exposure to 0.75% permethrin (WHO, 1998) was calculated by gender for each age cohort. There was a significant trend in survival with age in females (linear regression: P=0.04; R^2=0.59) and males (linear regression: P=0.02; R^2=0.60) in the FUMOZ-R strain. Although the R^2 value was close to 1 indicating a loose fit of the data, the P value was still significant. In both cases survival tended to decrease with age. Figure 2.6 shows the trend of survival across all the ages. Three-day old females showed a mean survival of 78.1%, which decreased steadily through ages 5 (71.71%), 10 (68%) and 14-days (37.70%), showing that female

susceptibility to permethrin increased with age. The lowest survival was recorded in the blood-fed female cohort at 14-days (32.70%), although a two sample *t*-test revealed that there was no significant difference between blood-fed and unfed females at this age (two sample *t*-test: *P*=0.64). Survival increased in the female cohorts at 20-days old (53.39%) and decreased again at 30-days (39.20%). A two sample *t*-test done between 3-day and 14-day old unfed females revealed that there was a significant difference in survival between the two ages (*P*=0.0006) (Table 2.7). A second two sample *t*-test between 3-day and 14-day old blood-fed females revealed that there was a significant difference in survival between the two ages (*P*=0.0011).

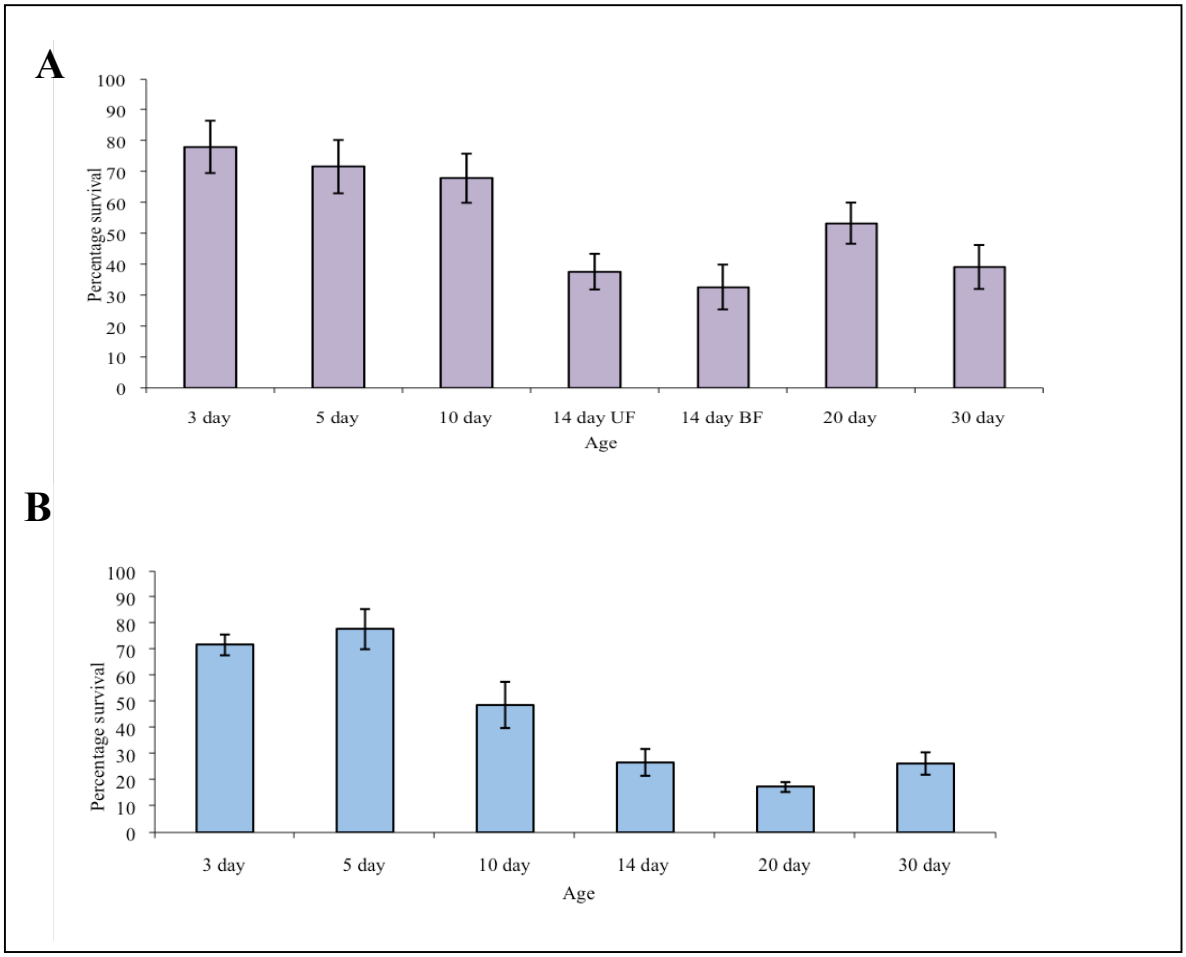

Figure 2.6 Percentage survival of 3 to 30-day old pyrethroid resistant *An. funestus*, FUMOZ-R, in (A) females and (B) males after exposure to 0.75% permethrin
14-day UF = unfed female mosquitoes
14-day BF = blood-fed female mosquitoes

A similar trend was observed in the males (Figure 2.6 B). The male's resistance to permethrin was high at ages three (71.81% survival) and five (77.86% survival). At 10-day old the percentage survival decreased to 48.83% and then steadily decreased to a low of 26.84% at age 14. However, the lowest survival in the males was recorded at age 20 (17.40%). The percentage survival increased to 26.40% at 30-days. There was a significant difference in percentage survival between 3-day and 14-day old males based on a two sample *t*-test (*P*=0.0000).

Table 2.7 Two sample *t*-test

Comparison	Mean	Replicate size	Standard deviation	Standard Error	*P*-value
3-day unfed vs. 14-day unfed females	21.886 62.309	10 25	26.828 28.832	8.4836 5.7664	0.0006
3-day vs. 14-day males	28.186 73.160	10 25	12.563 25.651	3.9726 5.1303	0.0000
3-day unfed vs. 14-day blood-fed females	21.886 67.286	10 9	26.828 22.943	8.4836 7.6476	0.0011
14-day unfed vs. 14-day blood-fed females	62.309 67.286	25 9	28.832 22.943	5.7664 7.6476	0.6445

2.5.2 qPCR mRNA levels analysis of *CYP6P9*, *CYP6P13* and the combined effect of both genes within the resistant colony, FUMOZ-R

qPCR was successful for all three gene sequences and the results were evaluated by a number of criteria before data analysis was complete. qPCR optimisation included determining the correct primer concentrations and the annealing temperatures for each of the genes. All reactions resulted in good exponential curves and C_Q values (Figure 2.7). The PCR efficiencies for all genes were between 90–105% (Figure 2.8). The R^2 value was greater than 0.995 and the slope of the regression line was always ± -3.350.

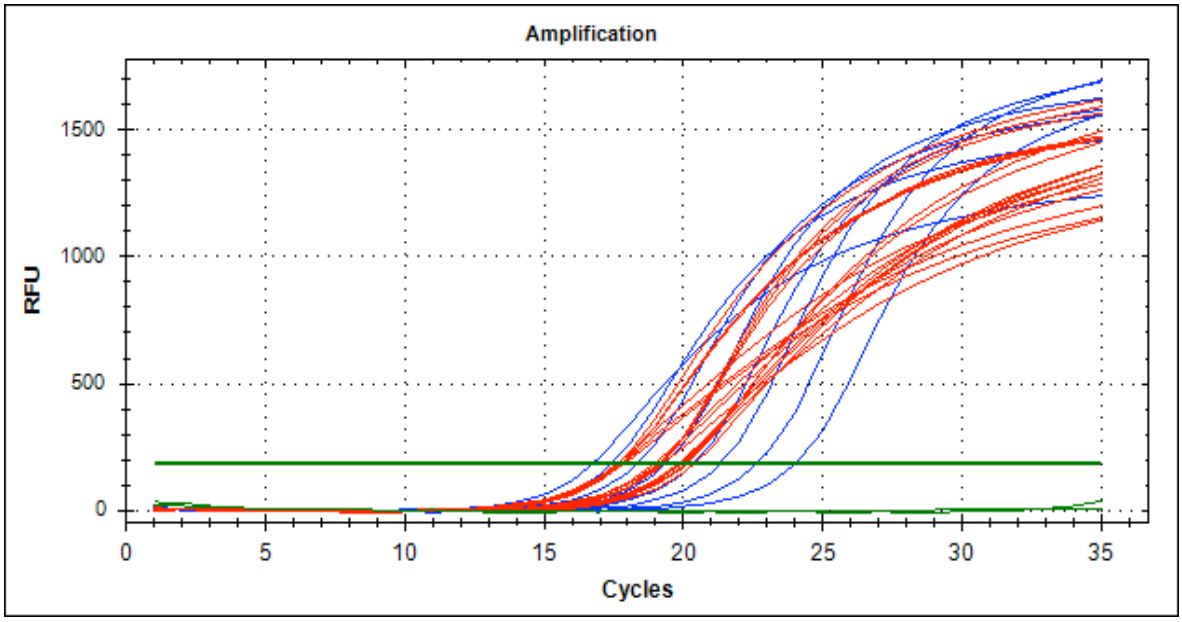

Figure 2.7 Amplification plot for the combined gene effect (*CYP6P9* and *CYP6P13*)
The amplification curves in blue represent the standard curves and the amplification curves in red are that of the samples of the combined gene effect (RFU- relative fluorescence unit)

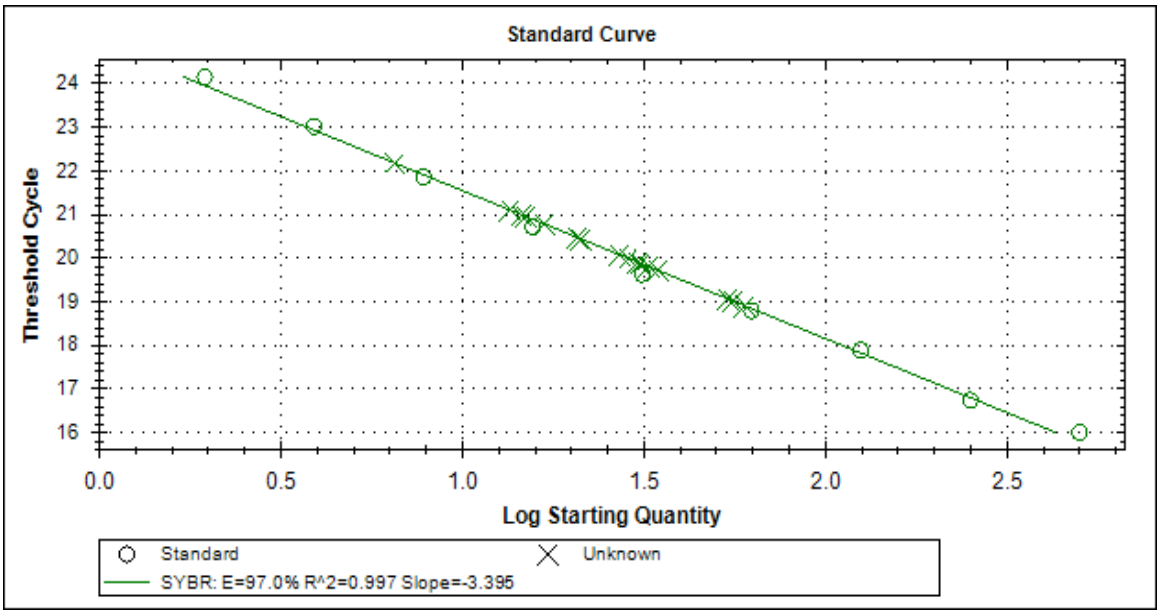

Figure 2.8 The standard curve of the combined genes, *CYP6P9* and *CYP6P13*
The PCR efficiency, R^2 value and the slope of the line are all represented in this graph

A melt peak analysis was also carried out to determine if any primer dimers had formed and if any contamination of the reaction had occurred. Any gene of interest would have a

single melt temperature and a different melt temperature detects any foreign samples present in the reaction.

2.5.3 mRNA levels analysis using the "combined *CYP6P9* and *CYP6P13* genes"

Amenya *et al.* (2008) reported that this gene (combined *CYP6P9* and *CYP6P13* genes) was 3.5-fold and 15-fold overexpressed in 3-day old males and females, respectively when the susceptible and resistant strains were compared. Wondji *et al.* (2009) on the other hand concluded that the gene (combined *CYP6P9* and *CYP6P13* genes) was 18-fold and 25-fold overexpressed in males and females respectively. The differences in expression values between these two studies can be attributed to dissimilar RNA extraction techniques used and the different generations of FANG and FUMOZ-R mosquito strains used. However, it is clear from both these studies that the combined gene was overexpressed. None of these authors looked at the change in gene transcription over age. In this section, the change in mRNA expression over age within the pyrethroid resistant strain was investigated.

The transcript copy number ratio (tCNR) of the "combined genes *CYP6P9* and *CYP6P13*" using the non-specific primers in FUMOZ-R females was calculated. tCNR varied between 1.3, 0.94, and 1.93 at age 3, 5 and 10-days, respectively. However, the tCNR decreased at day 14 to 1.18 (Figure 2.9A), and increased slightly to 1.35 in this age group in association with a blood meal although there was no significant difference in tCNR between blood-fed and unfed samples (two sample *t*-test: P=0.8). There was no significant difference between 3-day and 14-day old (P=0.52) nor between 10-day old and 14-day old unfed females (P=0.12). The tCNR was highest at day 20 (1.98) in females. The tCNR in males was lowest at day 3 (3.89) and highest at day 10 (15.27) (Figure 2.9 B).

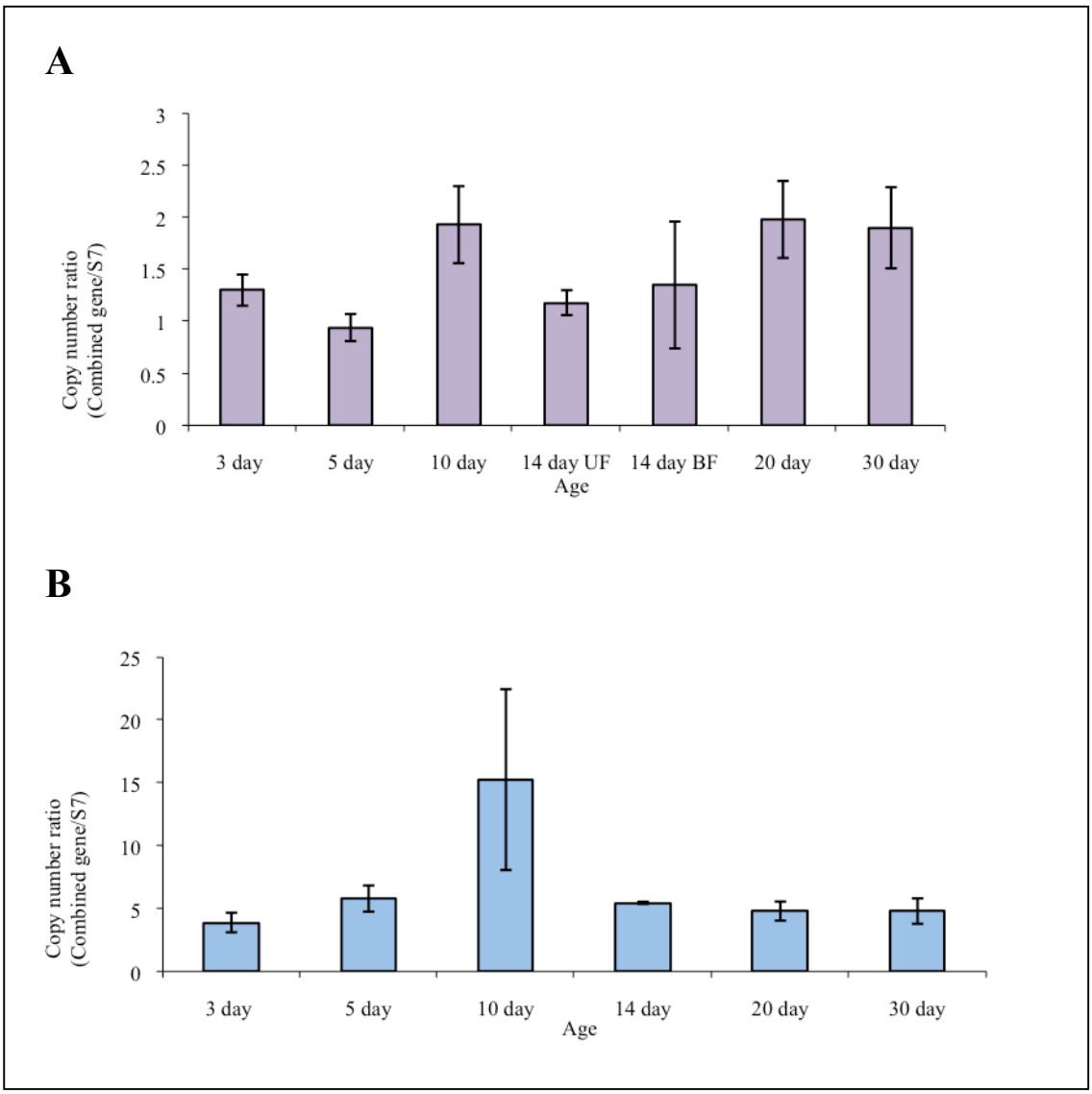

Figure 2.9 mRNA levels expressed as copy number ratio of the combined effect of *CYP6P9* and *CYP6P13* in (A) females and (B) males by age from the pyrethroid resistant *An. funestus* FUMOZ-R strain
14-day UF = unfed female mosquitoes
14-day BF = blood-fed female mosquitoes

2.5.4 mRNA expression of *CYP6P9*

We first established if *CYP6P9* was differentially expressed between the resistant and susceptible strains using gene-specific primers. Relative gene expression levels of *CYP6P9* were compared between 3-day old FUMOZ-R and 3-day old FANG strains. The relative expression of *CYP6P9* was 67-fold and 51-fold higher in 3-day old resistant FUMOZ-R

females and males respectively compared with the susceptible strain of the same age (Figure 2.10).

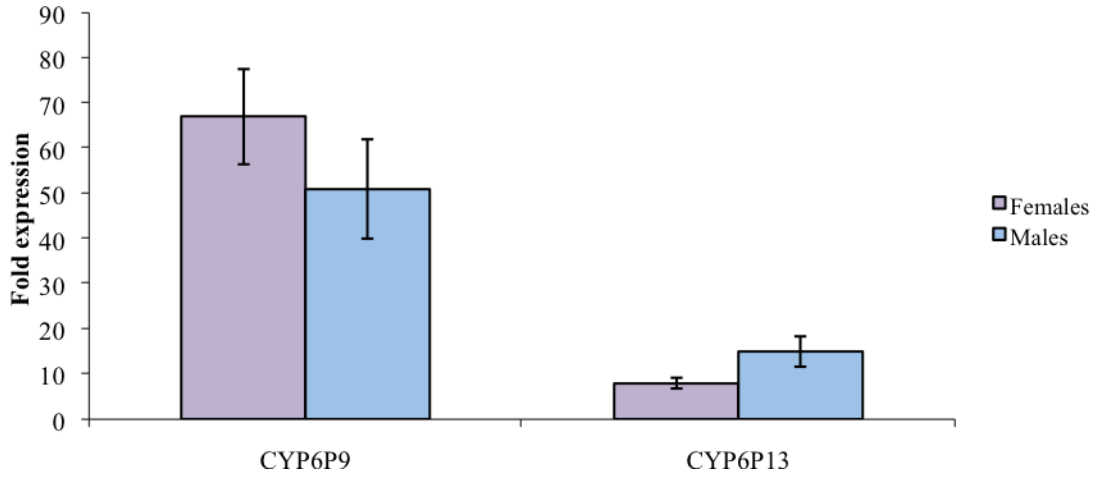

Figure 2.10 Fold overexpression of *CYP6P9* and *CYP6P13* in female and male 3-day old pyrethroid resistant *An. funestus* FUMOZ-R expressed as a ratio relative to the insecticide susceptible FANG strain

In order to correlate *CYP6P9* mRNA levels with insecticide survival over the range of ages within the resistant strain, absolute quantification analysis was used. Absolute quantification of this gene in the resistant FUMOZ-R strain showed a similar pattern to that of the combined gene effect. In the pyrethroid resistant females, as well as in the males, the tCNR was high at day 10 (9.3 for females and 20.1 for males) but decreased by day 14 (3.63 for females and 12.1 for males) (Figure 2.11 A).

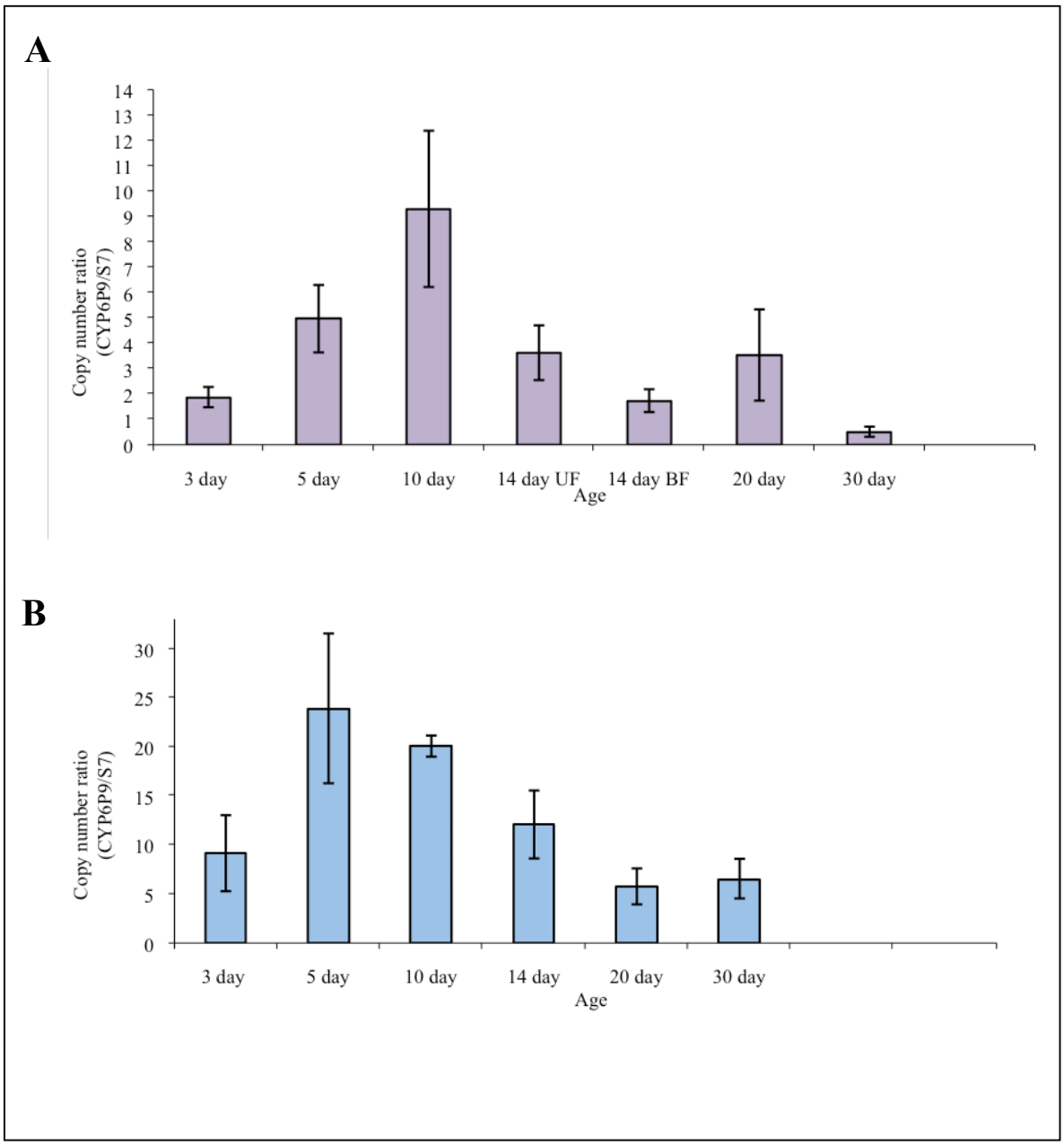

Figure 2.11 *CYP6P9* mRNA levels by age, expressed as copy number ratio, in pyrethroid resistant *An. funestus* FUMOZ-R (A) females and (B) males

In 3-day old females, the tCNR was 1.88, which increased to 9.3 by day 10. However, the tCNR decreased to 3.63 for unfed females and 1.74 for blood-fed females at day 14, although there was no significant difference in tCNR between blood-fed and unfed cohorts (two sample *t*-test: *P*=0.18). The tCNR increased again at day 20 (3.54) and then decreased to 0.52 at day 30.

Analysis of adult males showed that the tCNR was 9.15 at 3-days, which increased to 23.9 at 5-days followed by a steady decrease (20.1 at 10-days, 12.1 at 14-days, 5.77 at 20-days and 6.56 at 30-days) (Figure 2.11 B).

2.5.5 mRNA expression of *CYP6P13*

Primers for the *CYP6P13* gene were designed and tested briefly by Matambo (2008) on FANG cDNA and genomic DNA (gDNA) and FUMOZ-R cDNA and gDNA. The results from that study showed that the *CYP6P13* gene fragment could only be amplified in FANG gDNA. These primer sets were, therefore, re-tested on FANG and FUMOZ-R cDNA and gDNA. Primer set (1) had worked on both FANG and FUMOZ-R cDNA and gDNA, whilst primer set (2) and (3) only worked on FANG cDNA and gDNA but did not wok on FUMOZ-R cDNA and gDNA (Figure 2.12). Primer set (1) was, therefore, used in the qPCR studies. Due to only one primer set being required for this study, further optimization was not carried out on the other two primer sets.

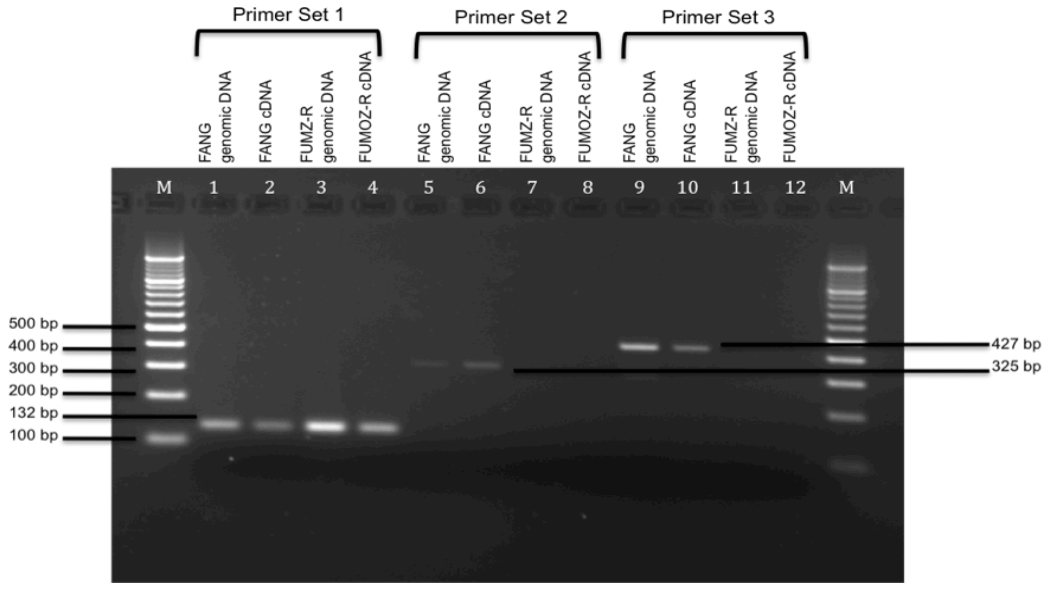

Figure 2.12 Amplification of a partial fragment of *CYP6P13* gene using FUMOZ-R and FANG cDNA and genomic DNA. The negative control was included in the PCR amplification reaction and loaded on another gel, however not present on this gel. M: Molecular weight marker

As mentioned above, we first established if *CYP6P13* was differentially expressed between the pyrethroid resistant and susceptible strains when using gene-specific primers as this had not been published by Amenya *et al.* (2008) or Wondji *et al.* (2009). Relative gene expression levels of *CYP6P13* were compared between 3-day old FUMOZ-R and 3-day old FANG strains. The relative expression of *CYP6P13* was 8-fold and 15-fold higher in the 3-day old resistant FUMOZ-R females and males respectively compared to samples of similar age from FANG (Figure 2.10).

mRNA levels of *CYP6P13,* using the absolute quantification method, within the resistant strain over age revealed that the tCNR changed only slightly from 4.69 in 3-day old females to 4.82 in 10-day old females, while males of the same age had a tCNR of 28.84 (Figure 2.13). However, there was a considerable change in tCNR between 3 and 14-day old unfed females although the difference is not significant (two sample *t*-test: P=0.15). The tCNR of the gene had decreased from 2.0 at 14-days when the females were not given a blood meal to a significant 0.38 when the females had access to a blood meal (P=0.01). The tCNR increased to 0.79 by day 30 in females and had increased to 13.67 in males from 8.92 at day 20.

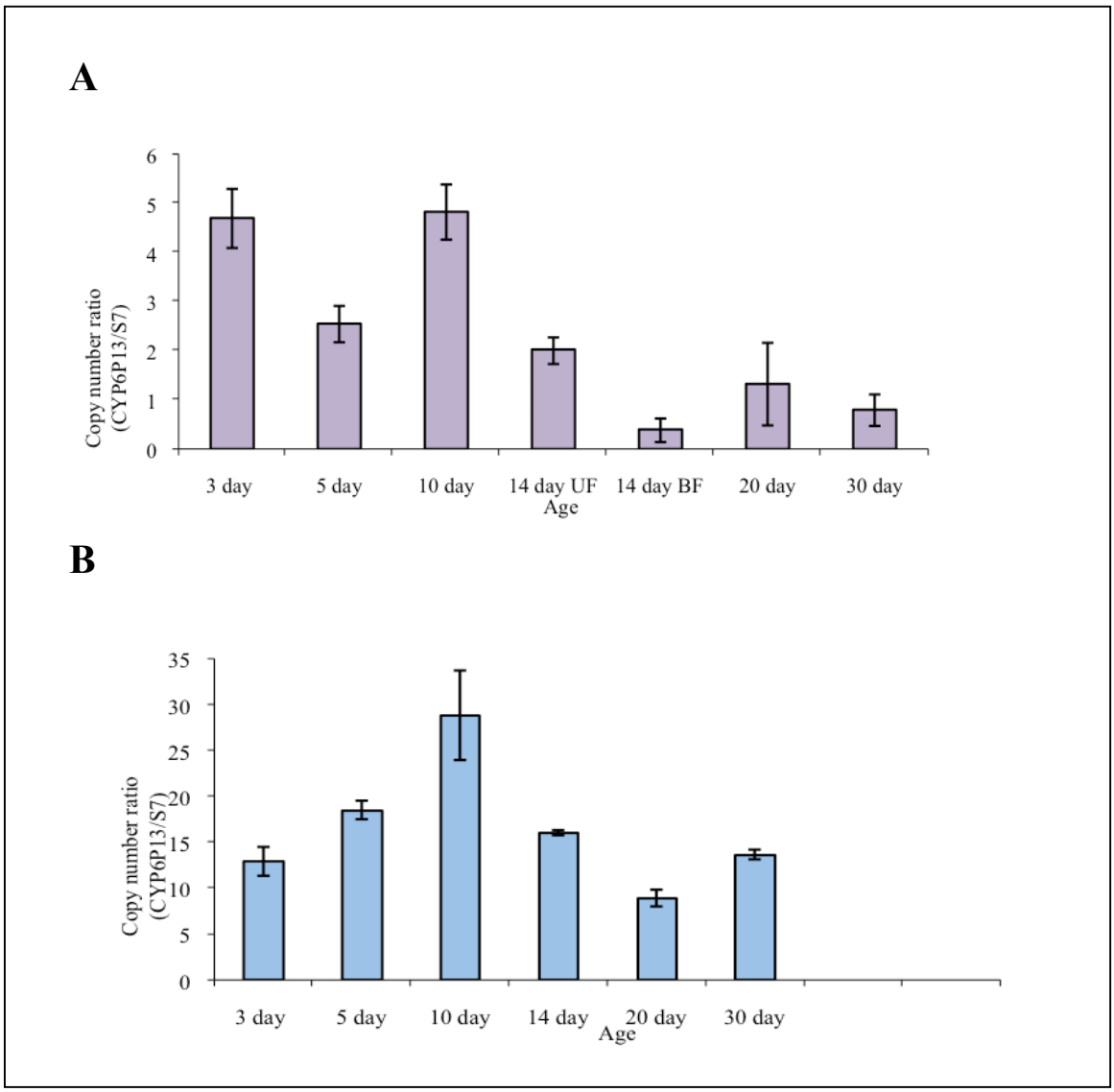

Figure 2.13 *CYP6P13* mRNA levels by age, expressed as copy number ratio, in pyrethroid resistant *An. funestus* FUMOZ-R (A) females and (B) males

2.5.6 Correlation between survival and mRNA levels

There was no correlation between percentage survival and tCNR of the combined effect gene in females (linear regression: P=0.60; R^2=0.15) or males (linear regression: P=0.90; R^2=0.24). Similar results were obtained for *CYP6P9* (linear regression: females, P=0.46; R^2=0.07; males, P=0.16; R^2=0.27) and *CYP6P13* (linear regression: females, P=0.06; R^2=0.52; males, P=0.52; R^2=0.11).

It was interesting to find that the percentage survival increased at day 20 and the tCNR for each gene also increased at this age. This suggests that these genes, together with other genes, could play a role in insecticide resistance. This could also imply that there is an association between phenotype expression and mRNA levels at this age.

2.5.7 Confirmation of qPCR products

All qPCR products were confirmed by sequencing. The *CYP6P9* gene was 250bp in size thus allowing for successful sequencing without cloning. The remaining genes, *CYP6P13*, the combined gene primer set and *rsp 7* genes were all < 140bp in length and were too small and as a result failed sequencing on two attempts. These genes were successfully cloned into the pGEM-T Easy Vector. The PCR conducted on clones for all genes worked on the first attempt (Figure 2.14). The size of the *CYP6P13* gene PCR product was approximately 132bp. The size of the T7 fragment length is 78bp and the SP6 fragment length is 100bp. Therefore, the size of the *CYP6P13* cloned PCR product together with the T7and SP6 fragments is ~ 310bp. The size of the *rsp 7* and the combined genes cloned PCR products were 313bp and 318bp, respectively. The size of the positive ligation cloned PCR products was 650-700bp in length.

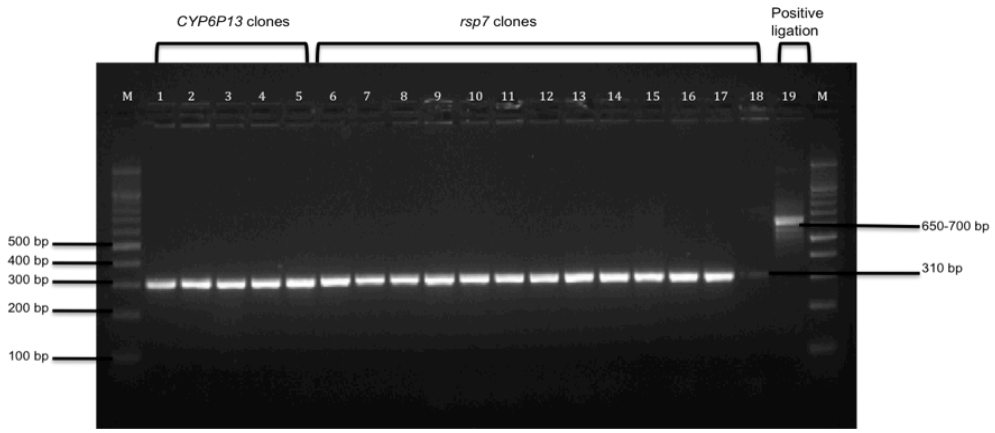

Figure 2.14 Amplification of selected clones of the *CYP6P13* and *rsp 7* genes
Lane M: Molecular weight marker; lanes 1-5: *CYP6P13* clones; lanes 6-18: *rsp 7* clones; lane 19: positive ligation clone. The negative control was included in the PCR amplification reaction and loaded on another gel, however not present on this gel.

The cloned products were sent to Macrogen (Korea) and sequencing was successful on the first attempt. An example of a portion of the sequence obtained is presented in Figure 2.15. A BLAST search (NCBI) was performed on all the sequences and all the genes had a percentage similarity > 98% to the genes of interest.

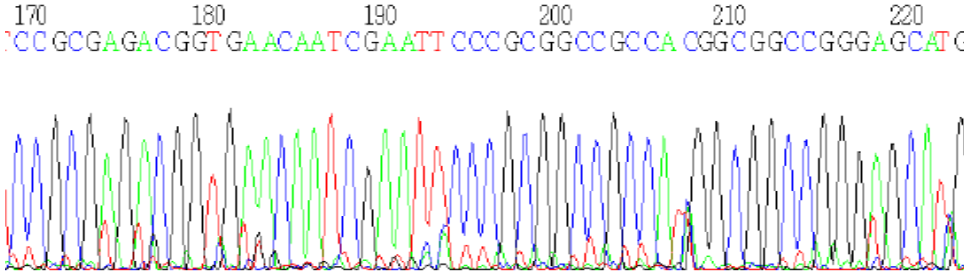

Figure 2.15 A portion of the chromatogram of the forward sequence of the *CYP6P13* gene

2.6 Discussion

Variation in the expression of insecticide resistance with age is shown here for *An. funestus* and has also been described in other anophelines. Hunt *et al.* (2005) describe fluctuations in the expression of pyrethroid resistance with age in unselected and selected generations of FUMOZ-R. Lines and Nassor (1991) reported that resistance to DDT declines in association with age in *An. gambiae.* The same phenomena has been found with pyrethroid tolerance in *An. stephensi* and *An. gambiae* (Hodjati and Curtis, 1999), resistance to malathion in *An. stephensi* (Rowland, 1985; Rowland and Hemingway, 1987) and *An. arabiensis* (Lines, 1983; Rawlings, 1985). The effect of age may lead to a weakening of resistance mechanisms as a consequence of a general progressive decrease in physiological capacity (Hodjati and Curtis, 1999). This could in turn result in the mosquitoes having reduced ability to respond or adapt to environmental changes.

The bioassay data revealed that there was a significant difference in percentage mortality (as per conversion from percentage survival) between the ages in the resistant *An. funestus* strain when exposed to permethrin. The data showed that mortality increased as the adults aged. Successive blood-feeding of females prior to 14-days of age did not affect female susceptibility to pyrethroids as assessed four days after the last blood meal when compared to unfed females of the same age. When using standard WHO susceptibility assays, however, pyrethroid resistance is enhanced in the FUMOZ-R strain four hours post blood-feeding in 3-day old females using dose response analysis (Spillings *et al*., 2008). Hunt *et al.* (2005) showed that pyrethroid induced mortality was significantly reduced in 14-day old and 20-day old blood-fed FUMOZ-R females when compared to unfed females.

These results collectively suggest that the level of the resistance phenotype varies in response to fluctuations in the physiological state of the mosquito, which is a function of various parameters including age, blood-feeding history and mating status. This information could impact on vector control as pyrethroid resistant females are likely to survive pyrethroid intoxication beyond 14-days of age, enabling infective individuals to transmit malaria even though the rate of mortality in this age cohort is higher. A different situation would have been possible if females were fully susceptible to pyrethroids from 14-days of age.

The overexpression of at least one P450 gene, *CYP6P9,* has been reported by two independent groups (Amenya *et al.,* 2008; Wondji *et al.,* 2009). However, there is no data to date showing whether or not this gene combination (*CYP6P9* plus *CYP6P13*) is able to metabolise pyrethroids, and the data given here show no correlation between resistance phenotype expression (as measured by bioassay) and mRNA levels. If the phenotype is

showing reduced susceptibility to pyrethroids with age it will most likely be correlated with a change in those genes associated with resistance, as demonstrated at day 20 in females.

Amenya *et al.* (2008) showed that *CYP6P9* is differentially expressed in 3-day old females and males (~15- and 3-fold, respectively) of the pyrethroid resistant FUMOZ-R strain. Wondji *et al*. (2009) confirmed that this gene is also differentially expressed in the same strain. However, both of these studies amplified *CYP6P13* or in combination with *CYP6P9* owing to sequence similarities between them. The present study attempted to elucidate the role of these genes in pyrethroid resistance individually and establish the molecular basis for age variation in the expression of resistance. *CYP6P9* and *CYP6P13* were both overexpressed in the resistant FUMOZ-R strain relative to the insecticide susceptible FANG strain, implying that these genes are associated with pyrethroid resistance in *An. funestus.*

Relative quantification revealed that *CYP6P9* had a 67-fold up-regulation in 3-day old FUMOZ-R females when compared to the susceptible strain. *CYP6P13* showed only a 8-fold overexpression in females. Both these genes showed either a much higher or lower fold expression than the 15-fold overexpression reported by Amenya *et al.* (2008) using samples of the same age. The discrepancy in the results is due to the fact that these genes were now examined individually and not as a combined gene. These results also suggests that *CYP6P9* and *CYP6P13* do not contribute cumulatively to the total fold effect reported by Amenya *et al*. (2008), and that they may not be the only genes that were amplified by Amenya *et al.* (2008) and Wondji *et al.* (2009). Due to sequence similarity of the P450 genes it is highly possible that other closely related P450 genes are co-amplified when

primers from Amenya *et al.* (2008) are used. Without a full genome sequence available it is difficult to explain the differences observed in the qPCR data. Genes within the same P450 sub-family, for example CYP6P, will share a >55% identity at the amino acid sequence level (Feyereisen, 1999). Fourteen CYP6 genes have been identified in *An. gambiae* (Ranson *et al.,* 2002b). Four CYP6P (1-4) genes were aligned and these showed a high level of similarity (Ranson *et al*., 2002b). It is, therefore, possible that the fold-over expression reported by Amenya *et al.* (2008) and Wondji *et al.* (2009) is the combined effect of four genes (assuming the same number of genes in the CYP6 class is present between these two species). A study conducted by Cuamba *et al.* (2010) on *An. funestus* field samples from Mozambique (Tihuquine) showed that *CYP6P9a* and *CYP6P9b* were overexpressed in both females and males. These genes were also differentially expressed when the Tihuquine samples were compared to FANG and a susceptible field samples from Mali. Morgan *et al.* (2010) also found the overexpression of *CYP6P9b* in females and males in the Tororo population when compared to FANG.

The bioassay data clearly showed that mortality to permethrin increased with age in the resistant strain. We analysed the tCNR of both *CYP6P9* and *CYP6P13* to investigate if there is an association between mRNA levels and permethrin mortality over age within the resistant strain. The highest tCNR was observed in *CYP6P9* (9.30) and *CYP6P13* (4.82) at 10-days old. Permethrin survival of females was also the highest between 3-10 days of age. Overall *CYP6P9* and *CYP6P13* showed decreased absolute mRNA levels over time and this was mimicked by bioassay data although the difference was not significant. As indicated above it is most likely that these are not the only genes involved and that they may function in a cascade manner to detoxify pyrethroids at various stages in the adult

female life cycle, a function that is most likely assisted by a number of other gene products.

Absolute mRNA levels (given as copy number ratio) in the combined gene effect (*CYP6P9* and *CYP6P13*) did not alter significantly in association if females had access to blood meals, but when these genes were assessed separately the absolute mRNA levels were reduced following blood-feeding.

Overall, the male tCNR's were higher than those of females, in contrast to the bioassay data in which males showed a slightly lower survival than females. This discrepancy may be attributable to other biological functions (such as pheromone production) that P450s are associated with in males (Kasai and Tomita, 2003). This will need to be investigated further.

This study has shown the effect of permethrin exposure at different ages in *An. funestus* mosquitoes. Permethrin tolerance generally decreases with age. This part of the study also highlighted two important genes that are associated with permethrin resistance in adults. Both of these genes show variation in mRNA levels with age. As so little is known about their role in pyrethroid metabolism, it is possible that they are only a link in a chain of reactions that result in the pyrethroid resistance in this species. Other possible metabolic genes and physiological events in addition to these two genes may contribute to the production of the resistance phenotype.

CHAPTER THREE

Microarray analysis of pyrethroid resistant *Anopheles funestus*, from southern Africa*

3.1 Background

Expression profiling between different sample types was challenging until the advent of the microarray technology. Microarrays enables one to study thousands of genes simultaneously. For example, they can be used to compare gene expression levels between normal and diseased tissues, help detect single nucleotide polymorphisms (SNPs), and enable comparisons between different life stages or treated versus untreated time courses (Korkola *et al.,* 2003; Naidoo *et al.,* 2005).

3.1.1 What are microarrays?

Microarray technology evolved from Southern blotting where fragmented DNA is applied to a substrate, which is then probed to a known gene. In 1995, Patrick Brown and colleagues from Stanford University developed the first microarray slide to conduct gene expression studies (Naidoo *et al.,* 2005). A microarray is an orderly arrangement of thousands of sequenced and identified genes printed on silicon chips, nylon membranes or a glass surface onto which complementary DNA (cDNA) or gene specific oligonucleotides can be spotted (Yang *et al.,* 2005). The DNA microarray slide may contain the entire genome of an organism or a group of genes of particular interest. There are two types of microarrays. One consists of probes of a known sequence of up to 70 mers of cDNA or oligonucleotides. The probes are spotted robotically onto a glass surface. The second type

* Footnote: Accepted for publication in Journal of Pesticide Biochemistry and Physiology. See Appendix D, page 149.

is the *in situ* synthesized oligonucleotide array, the Affymetrix GeneChip system. In this system, the DNA oligonucleotides are synthesized *in situ* onto the DNA chip and this is done by photolabile protecting groups and photolithographic masks (Lipshutz *et al.,* 1999). These Affymetrix arrays consist of oligonucleotides that are 25 base pairs in length.

3.1.2 Microarray experimental design

Microarray experiments consist of three different categories, which assist the researcher to determine their objective/s and these are class comparison, class discovery and class prediction (Simon and Dobbin, 2003; Naidoo *et al.,* 2005).

i. Class comparison

A class comparison design involves comparison between two different samples, for example differential gene expression between a susceptible and a resistant mosquito.

ii. Class prediction

Class prediction involves using the gene expression data obtained by class comparison and then using a multi-gene formula to determine to which class a new sample belongs.

iii. Class discovery

Class discovery involves studies in which the samples are not predetermined to different classes prior to the microarray experiment. This involves discovering clusters of the samples based on gene expression data and then identifying the genes that distinguish these clusters.

Before any microarray experiment is carried out, the study design needs to be established. There are four types of designs (Figure 3.1). These include the direct comparison design, reference design, balanced block design and loop design (Simon and Dobbin, 2003; Naidoo *et al.,* 2005).

i. The direct comparison

This design (Figure 3.1A) is used to compare two samples e.g. the susceptible RNA sample is co-hybridised to the resistant sample on the same slide or untreated to the treated sample (Naidoo *et al.,* 2005).

ii. The reference design

The reference design (Figure 3.1B) is an indirect method in microarray experiments (Churchill, 2002). This design uses a common aliquot of reference RNA on every array, i.e. each experimental sample is hybridised against a common reference sample. The intensity of the spot of the sample of interest is measured or standardized against the same spot on the same array for the reference sample (Simon and Dobbin, 2003). The reference RNA sample usually has to be available in large quantities (Naidoo *et al.,* 2005). This design can be used when one wants to determine differentially expressed genes in two different transgenic sample types. The untransformed sample, transgenic sample type 1 and transgenic sample type 2 can be individually compared to a reference sample. The reference sample consists of a pool of equal amounts of RNA from each sample. The reference sample has to be labelled with the same dye in each experiment (Naidoo *et al.,* 2005).

iii. The balanced block design

This design (Figure 3.1C) usually compares two groups of samples to each other. For example, if one wishes to compare four insecticide susceptible mosquito samples to four resistant samples, then the array is set up as follows: on one half of each array a resistant sample will be compared to the susceptible sample where the resistant sample is labelled with red dye and the susceptible sample labelled with green dye. The labelling of the samples will be reversed on the other half of the slide (Simon and Dobbin, 2003; Naidoo *et al.,* 2005). A disadvantage of this method is that cluster analysis of the expression profiles cannot be performed effectively. One advantage of this method is that half the number of slides can be used as compared to the reference or direct comparison (Naidoo *et al.,* 2005).

iv. The loop design

The loop design (Figure 3.1D) links samples together in a loop in an array hybridisation. The sample comparisons control for variation in spot size and sample distribution patterns using a statistical model (Simon and Dobbin, 2003). In this design, two aliquots of each sample and *n* arrays are used to study *n* samples. For example, a comparison of three, five and 10-day old mosquitoes would enable determination of gene expression differentiation by age. It is usually recommended that dye swaps are performed using the same sample (technical replicates) or a balanced block design is performed by carrying out a loop design with the biological replicates labelled with the reverse dyes to account for dye biases (Naidoo *et al.,* 2005). This design results in half the variance per estimate, because each sample occurs twice, rather than once, at the cost and use of only one chip.

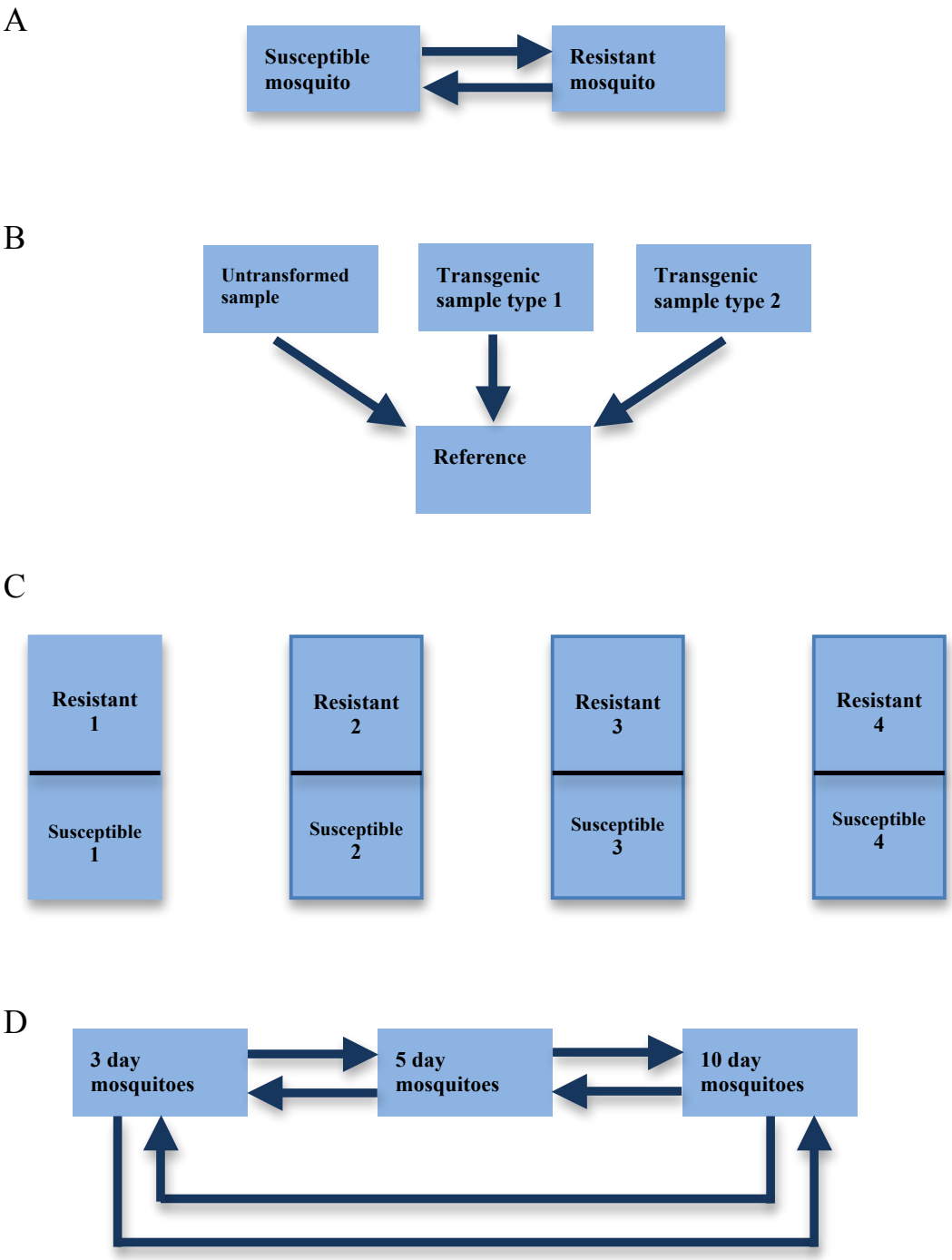

Figure 3.1 Diagrammatic representations of the four microarray designs
An arrow represents each microarray slide. The head of the arrow indicates the sample labelled with the red (Cy-5) dye and the tail labelled with the green (Cy-3) dye. (A) The direct comparison between a resistant and susceptible sample. (B) The reference design using a common reference sample. (C) A balanced block design showing the comparison of four resistant samples to four susceptible samples. (D) A loop design compares all samples to each other (Naidoo *et al.,* 2005)

3.1.3 The microarray technique

The microarray technique consists of four steps, the first involves manufacturing of the microarray slide (Butte, 2002). This entails a chip or glass slide with a special surface chemistry. The slide is then spotted with the genes/probes of interest. Probe spotting is carried out by robotic arms with fine needles. These are inserted into wells containing the probes, and the probes are then spotted onto the slide to create a positional grid. The position of each probe is usually determined prior to printing. Control spots are also spotted onto a slide. These control spots consist of blank spots containing no probes, calibration controls, which contain equal amounts of Cy-3 and Cy-5 dye and ratios spots, which contain varying amounts of Cy-3 and Cy-5 dye. Post hybridisation, the control blank spots should contain no colour, the calibration controls should be yellow and the ratio spots should be varying shades of green or red depending on the amounts of Cy-dye added. The control spots indicates how well the labelling or microarray experiment was carried out.

The second step is sample preparation (Figure 3.2). This requires RNA extractions, and cDNA/mRNA synthesis of the samples. The samples that are to be compared to each other are labelled with either a Cy-5 (red) or a Cy-3 (green) dye. These samples are then mixed together and cleaned using purification kits. The cDNA concentration and the Cy-dye incorporation are detected to check the quality of the material that is to be hybridised to the slide. The third stage involves washing of the slides. The slides need to be subjected to a series of washing steps in specially prepared wash solutions for pre- and post-hybridisation. This allows for the preparation of the slides for pre-hybridisation and the removal of unhybridised probes in the post-hybridisation step. After the pre-hybridisation treatment of the slides, the samples are deposited onto the arrays and incubated overnight for ± 16 hours. Usually two technical and three biological replicates are carried out. Dye

swaps are also considered a form of technical replication. Here, the hybridisations are carried out with the reciprocal dye labelling of sample targets in the second hybridisation. Dye swaps reduce the dye bias in the red and green intensities. This form of technical repeat can only be included if enough sample material is available.

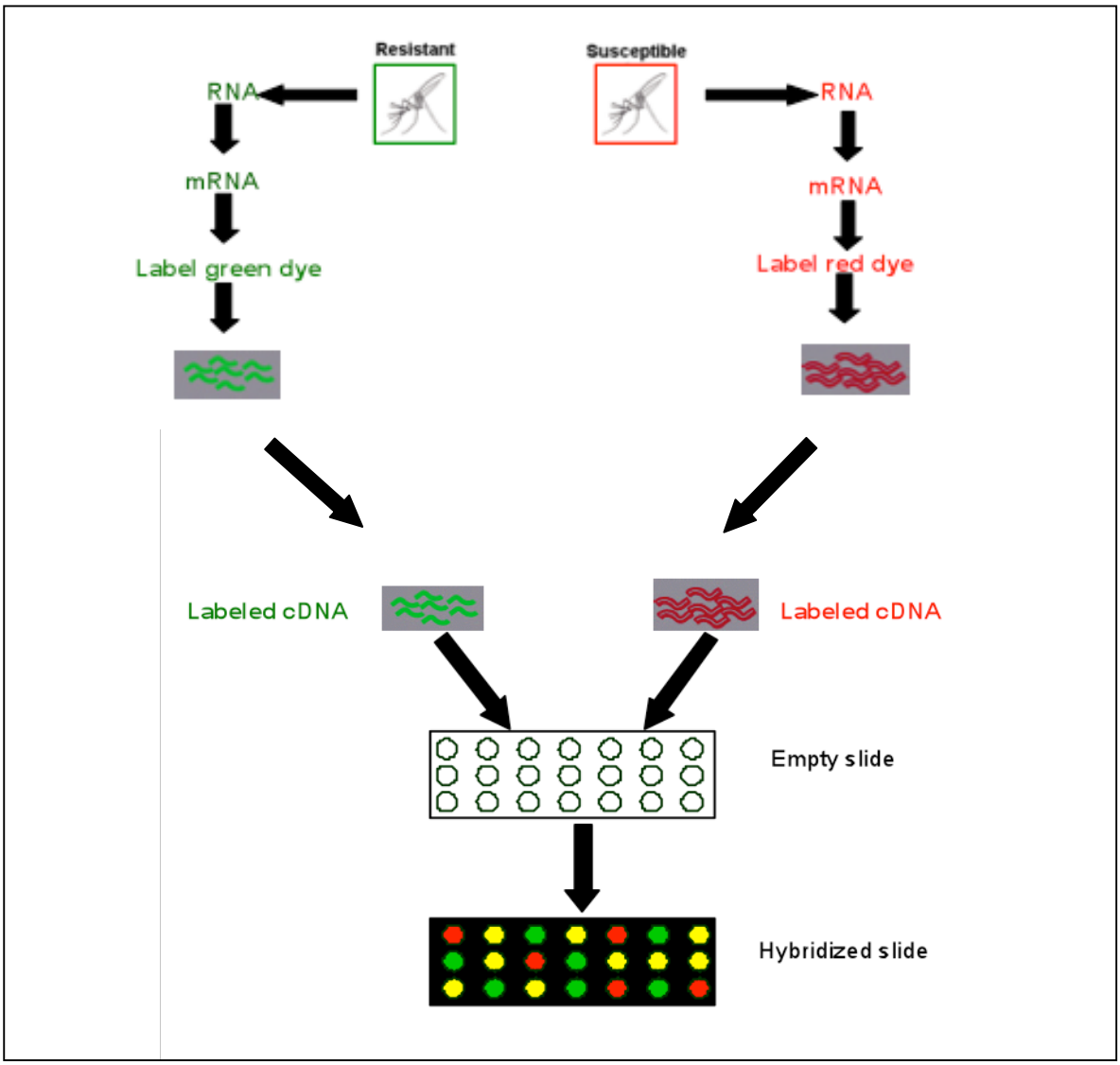

Figure 3.2 Workflow of a microarray experiment

The final stage involves scanning of the slides and data analysis. The slides are scanned under standard conditions using a specialised scanner. The features are edited in software specific for microarray data analysis. Spot finding is carried out and this involves 'flagging' the poor-quality features or spots (Bajcsy, 2005). The image is quantified into numerical values, these values are then run through software packages which allow the data to be analysed and interpreted. The microarray software packages TM4 (Saeed *et al.,* 2003) and Limma (Smyth, 2005) are generally used to carry out the analysis. Normalization (van Heerden *et al.,* 2007), background correction and statistical analyses are carried out on the microarray data.

3.1.4 Standardizing of microarray data

Data can be stored in different formats. This is sometimes problematic when data are presented and exchanged. The presentation of microarray data is now standardised by listing prerequisites. The Minimum Information About a Microarray Experiment (MIAME) is one such checklist that describes what information is essential if the experiment is repeated and interpreted (Brazma *et al.,* 2001). The MIAME checklist lists the following details as essential when sharing data: the raw data file for each hybridisation experiment (e.g. GPR files); the final normalised gene expression data; sample annotation including experimental factors and their values; experimental design; probe information on the microarray slide (e.g. oligo or cDNA probes, control spots); and the laboratory and data analysis protocols used (Brazma *et al.,* 2001).

3.2 Use of Microarrays to Determine Differentially Expressed Detoxification Genes in *An. funestus*

Very few studies have been carried out to determine the genes involved in detoxification in insecticide resistant *An. funestus* mosquitoes. Metabolic resistance (detoxification enzyme-based resistance) in insects is characterized by increased activity of detoxifying enzymes such as esterases, cytochrome P450 monooxygenases (cytochrome P450s), and glutathione *S*-transferases (GSTs) (Brogdon and McAllister, 1998; Liu *et al.,* 2006). All three enzyme groups are encoded by large gene families and identifying the individual genes associated with resistance can be laborious. This task was expedited for *An. gambiae* by the development of a custom microarray specifically designed to detect transcription variation in genes associated with insecticide resistance (David *et al.,* 2005). This array was named the detoxification chip. The array contains over 255 putative genes. A total of 103 of these genes belong to the cytochrome P450 monooxygenase (P450s), 41 are Red/Ox genes, 35 glutathione *S*-transferases (GSTs), 31 esterases, 5 ATP-binding-cassette transporters, and the remaining are control genes, tissue-specific genes and house-keeping genes (David *et al.,* 2005). David *et al.* (2005) used the chip successfully and identified a number of genes up-regulated in DDT and pyrethroid resistant strains of *An. gambiae* from East Africa. Some of these genes were previously implicated in insecticide resistance while others were novel, not previously associated with insecticide resistance (David *et al.,* 2005). The detox chip has been utilized for screening *An. gambiae* populations from Kenya and Zanzibar (David *et al.,* 2005), Benin (Djouaka *et al.,* 2008), Nigeria (Awolola *et al.,* 2009) and Ghana (Müller *et al*., 2007; Müller *et al*., 2008b) and has also been successfully used for *An. arabiensis* from Cameroon (Müller *et al.,* 2008a) and *An. stephensi* from Dubai (Vontas *et al.,* 2007).

In this study, we aimed to use the *An. gambiae* detox chip on *An. funestus* samples, which represents a cross-species hybridisation. Cross-species hybridisations are common and many studies have successfully identified differentially expressed genes using this method (see section Section 3.7). Many factors were considered prior to the implementation of this method (Bar-Or *et al.,* 2007). The percentage sequence identity between the reference species i.e., *An. gambiae* (the sample represented on the microarray) and the target sample i.e., *An. funestus* (the sample under study) was determined. The question of whether the use of cross-species hybridisation would yield the same results as same-species hybridisation was taken into consideration. Grigoryev *et al.* (2005) and Bar-Or *et al.* (2007) both conducted such studies and found that data sets from both the cross-species hybridisation and same-species hybridisation produced similar results. The reproducibility of the slides was also considered. Biological and technical replicates were carried out to ensure that the results obtained were consistent. The validation of the microarray results were confirmed by the use of other techniques such as quantitative PCR (qPCR) (Bar-Or *et al*., 2007).

3.3 Aim and Objectives

To date very few studies have been conducted to determine genes potentially involved in insecticide resistance in. *An. funestus.* The study conducted by Amenya *et al,* (2005) isolated 31 P450 genes and showed that *An. funestus* P450s have an average of 75% amino acid identity to that of *An. gambiae*. The *An. gambiae* detox chip was, therefore, used in this study as a tool to screen for differentially expressed detoxifying genes in both susceptible and pyrethroid resistant *An. funestus* strains. Selected significantly overexpressed genes from the microarray results were validated using quantitative PCR (qPCR).

3.4 Biological Material

3.4.1 Mosquito strains

Two *An. funestus* strains were used in this study. The pyrethroid resistant FUMOZ-R strain and the insecticide susceptible FANG strain. Information on the two strains is detailed in Chapter Two (Section 2.3).

3.5 Methods

3.5.1 Sample preparation and microarray hybridisations

Female and male *An. funestus* adults were separated on the day of emergence and fed on a 10% sugar solution until they were 3-days old. The detox gene transcription levels in the 3-day old resistant, FUMOZ-R, adult females were compared to insecticide susceptible females of the same age. The same was done for the males. The resistant strain was not

exposed to insecticides prior to RNA extractions. This strain has been under selection for generations to maintain high resistance levels. Each microarray comparison in all experiments consisted of three independent biological and two technical repeats which consisted of dye swaps to control for dye bias. A biological replicate involved isolating RNA independently from three sets of mosquito samples. The biological replicates served to control for biological diversity. The technical replicate involved carrying out the same experiment using the same sample on a number of occasions. The technical replicates controlled for technical variability in an experiment.

3.5.2 RNA isolation

Isolation of RNA was conducted using the PicoPure® RNA Isolation kit (Arcturus, KIT0204, USA). Extraction buffer (100µl) was added to 15 mosquitoes. These samples were homogenised and incubated at 42°C for 30 minutes in a heating block. The samples were then centrifuged at 12 000 rpm for two minutes, the supernatant removed and placed into a new tube. A volume of 100µl of 70% ethanol was added to the cell extract and mixed thoroughly. A RNA purification column was pre-conditioned by adding 250µl conditioning buffer onto the column filters membrane. This was left to incubate for five minutes and the column was then centrifuged at 14 000 rpm for one minute. The sample and ethanol mix was added onto the pre-conditioned purification column and centrifuged for two minutes at 270 rpm, immediately followed by a centrifugation at 14 000 rpm for 30 seconds. A volume of 100µl wash buffer 1 was added into the purification column and centrifuged for one minute at 9 900 rpm. The samples were DNase treated by adding 5µl DNase I Stock Solution and 35µl buffer RDD and left to incubate at room temperature for 15 minutes. The columns were centrifuged at 9 900 rpm for 15 seconds. Wash buffer 2 (100µl) was added into the purification column and centrifuged for one minute at 9 900

rpm. Another 100µl of wash buffer 2 was added into the purification column and centrifuged for two minutes at 14 000 rpm. The flow through waste was discarded and the column re-centrifuged for one minute at 14 000 rpm. The purification column was added to a new 0.5 ml microcentrifuge tube and 30µl of elution buffer was added directly onto the membrane of the purification column. The column was incubated at room temperature for one minute and centrifuged for one minute at 3 500 rpm to distribute elution buffer in the column, and then for an additional one minute at 14 000 rpm to elute the RNA. The quantity of RNA was determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA). The RNA was stored at -70°C until further use.

3.5.3 Synthesis of amplified mRNA

Complementary DNA (cDNA) and amplified mRNA (amRNA) were obtained using the RiboAmp^{TM} RNA Amplification Kit (Arcturus, KIT0201, USA). All primers and reagents were available from the kit. For the first strand (cDNA) synthesis total RNA (10µl) was added to 1µl of primer A. This was incubated at 65°C for five minutes and then cooled at 4°C for at least one minute. The complete 1st strand master mix (7µl) and 1st strand enzyme mix (2µl) was added to the RNA mixture and incubated at 42°C for 45 minutes followed by 4°C for at least one minute. To this, 2µl of 1st strand nuclease mix was added and the samples were incubated at 37°C for 20 minutes, 95°C for five minutes and then held at 4°C.

For the 2nd strand synthesis primer B (1µl) was added to the samples and incubated at 95°C for two minutes, then chilled and maintained at 4°C for at least two minutes. The complete 2nd strand synthesis mix (29µl) and 2nd strand enzyme mix (1µl) were added to the samples and incubated at 25°C for five minutes, 37°C for ten minutes, 70°C for five minutes and

4°C for two minutes. These samples were cleaned using the cDNA purification kit. The DNA binding buffer (DB) (250µl) was added to the DNA/RNA purification column for five minutes at room temperature and centrifuged for one minute. The second strand synthesis mixture was added to 200µl of DB, which was transferred into the purification column. The column was centrifuged at 270 rpm for two minutes, immediately followed by a centrifugation at 11 000 rpm for 30 seconds to remove flow through. DNA wash buffer (DW) (250µl) was added to the column and centrifuged at 14 000 rpm for two minutes. The flow through was discarded and the column placed into a clean centrifuge tube. DNA elution buffer (DE) (16µl) was placed onto the centre of the purification column membrane and centrifuged at 3 500 rpm for one minute, followed immediately by 14 000 rpm for one minute. The elution was retained for further processing.

In vitro transcription (IVT) was then carried out. To the cleaned samples, the following reagents were added for the IVT reaction: 8µl of IVT buffer, 12µl of IVT master mix and 4µl of IVT enzyme mix. These samples were incubated at 42°C for four hours. DNase mix (2µl) was added to each sample and incubated at 37°C for 15 minutes. These samples were cleaned using the amplified RNA (amRNA) purification kit. A volume of 250µl of RNA binding buffer (RB) was added to the DNA/RNA purification column, incubated for five minutes at room temperature and centrifuged at 14 000 rpm for one minute. RB buffer (200µl) was added to the IVT samples and pipetted into the purification column and centrifuged at 270 rpm for two minutes, immediately followed by a centrifugation at 11 000 rpm for 30 seconds. The RNA wash buffer (RW) (200µl) was added to the purification column, and centrifuged at 11 000 rpm for one minute. A total of 200µl of fresh RW buffer was added to the purification column, and centrifuged at 14 000 rpm for two minutes. The purification column was placed into a new 0.5 ml micro centrifuge tube provided in the kit

and 30µl of RNA elution buffer (RE) was added directly onto the centre of the purification column membrane. The columns were incubated at room temperature for one minute and centrifuged at 3 500 rpm for one minute, immediately followed by 14 000 rpm for one minute.

The amRNA was measured using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA) and the quality of the amRNA was assessed on a 0.8% agarose with 1x RNase-free TAE buffer. The expected yield of amRNA should be between 30 and 50µg and the A260/A280 ratio should range between 1.8-2.0. A concentration of 0.5µg of amRNA was mixed with 3µl DEPC-treated H_2O and 2µl loading buffer and denatured at 70°C for five minutes prior to loading the gel. The amRNA should appear as a smear on the gel from 250 to 1 800 bases in length, with the majority of product around 600 bases in length.

3.5.4 Microarray experiment

A RNA and primer mix which consisted of 8µg of amRNA for each of the samples, 2µl mRNA spike mix (Universal Lucidea Scorecard, Amersham), 1.3µl random hexamers (Invitrogen, 48190011, USA) and distilled water was incubated for five minutes at 70ºC. The RNA mix was reverse transcribed into Cy-labelled cDNAs using 2µl Cy-3 (GE Healthcare, PA53022, UK) or Cy-5-dUTPs (Amersham, PA55022, UK), 4µl 5x RT buffer, 2µl DTT, 0.4 µl dT-NTPs, 0.7µl RNAsin and 1.5µl Superscript III (Invitrogen, 18080044, USA). The samples were incubated at 50ºC for 2 ½ hours. A mixture of 1M NaOH/ 20mM EDTA (1µl) was added to the labelled cDNAs to stop the reaction. The Cy-labelled cDNAs that were to be hybridised together were pooled and purified using the Cyscribe™GFX™ Purification kit (GE Healthcare, 27960601, UK). Capture buffer

(500µl) was added to each Cyscribe column. The labelled samples were added to the buffer, mixed thoroughly and centrifuged at 13 000 rpm for 30 seconds. The flow through was discarded and 600 µl of wash buffer was added to each column and spun at 13 000 rpm for 30 seconds. Three washes were done per sample. The columns were transferred to a clean microcentrifuge tube and 60µl of elution buffer was added to each column, allowed to stand at room temperature for four minutes and centrifuged at 13 000 rpm for two minutes. Another 40µl of elution buffer was added to each column, allowed to stand at room temperature for four minutes and centrifuged at 13 000 rpm for two minutes.

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 not used in the hybridisation step. Approximately 2µl (5µg) of poly dA oligo (Amersham Biosciences, 277836, USA) was added to the purified targets to prevent nonspecific hybridisation. The labelled samples were dried in a speed vacuum centrifuge for one hour at 30ºC and re-suspended in 15.5µl of hybridisation buffer (Corning, 40026, USA). The targets were denatured by incubation at 95°C for five minutes. The *An. gambiae* detox chip was pre-treated using the Universal Hybridisation kit (Corning, 40026, USA), according to manufacturer's instructions. However, the wash solutions were made up using three times the amount of components as stated in the original *An. gambiae* protocol, after optimization (David *et al.,* 2005) (Table 3.1). The Pronto! Universal Pre-Soak Solution and Pronto! Universal Pre-Hybridisation Solution (50ml) were heated to 42ºC for 30 minutes. Sodium Borohydride (500 µl) was added to the Pronto! Universal Pre-Soak Solution and left to dissolve for five minutes. The slides were incubated in the solution at 42ºC for 20 minutes. The slides were rinsed in two consecutive

wash solutions 2 and incubated at room temperature for 30 seconds. The slides were incubated in the Pronto! Universal Pre-Hybridisation Solution at 42ºC for 15 minutes followed by a wash in wash solution 2 for one minute. This was followed by washing the slides in two consecutive wash solutions 3 at room temperature for 30 seconds. The detox chip was rinsed in deionised water for two seconds and dried by centrifugation at 2 500 rpm for 2 ½ minutes. The slides were stored in a dust/light/humidity free environment. The target mix was added to the microarray chips, which were placed into hybridisation chambers. The chambers containing the slides were placed into a water bath set at 38°C for 16 hours.

Table 3.1 Wash solution adjustments for the optimization of *An. funestus*

	Reagents	***An. gambiae* protocol**	***An. funestus* protocol**
Wash Solution 1 (250ml)	Universal wash reagent A	25ml	75ml
	Universal wash reagent B	1.25ml	3.75ml
	Deionised water	223.75ml	171.25ml
Wash Solution 2 (500ml)	Universal wash reagent A	25ml	75ml
	Deionised water	475ml	425ml
Wash Solution 3 (500ml)	Wash solution 2	100ml	300ml
	Deionised water	400ml	200ml

After incubation, the slides were washed using the Universal Hybridisation kit (Corning, 40026, USA). 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 five 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 2 500 rpm for two minutes.

3.5.5 Microarray scanning and data analysis

The slides were scanned using the Genepix 4000B scanner (Molecular Devices, USA). The PMT (photomultiplier tube) 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 analysed using the Limma software package version 2.12.0, from the BioConductor project, in the R programming environment (Smyth *et al.,* 2005). Image plots were generated to inspect the variation of background values across the arrays. Background correction was carried out using the 'normexp' method (Ritchie *et al.,* 2007) 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 (Smyth and Speed, 2003). 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 (Smyth, 2004). A top table was generated which revealed the highly differentially expressed genes for both the strains of interest. The *B*-statistics values were ranked from the highest to lowest for all the genes. The *B*-statistics value is the log odds that a gene is differentially expressed. If a gene has a *B*-statistic value of 0 it indicates that the gene has a 50% chance that it is differentially expressed. B values and *P*-values usually rank the genes in the same order. A high *B*-statistics value and a low *P*-value indicates that a gene is significantly differentially expressed. Genes exhibiting adjusted *P*-values <0.001 and fold-changes ≥1.5 were considered as statistically significant overexpressed genes.

The data were deposited into Vectorbase, a database where mostly molecular data or information on invertebrate vectors of disease is deposited. This facility allows one to browse genomes, deposit microarray and gene expression data and conduct BLAST searches (https://www.vectorbase.org).

3.5.6 Quantitative Real-Time PCR (qPCR)

Quantitative PCR analysis was carried out on 3-day old FUMOZ-R and FANG females and males to verify the results obtained from the microarray experiments. Only the top three genes common to both females and males were carried forward to qPCR analysis. qPCR on the remaining up-regulated genes will be carried out in future studies. Primers were designed for genes *CYP6M7* and *COI*. The primer pair used for the *CYP6P9* and *CYP6P13* genes was the gene-specific primers designed in Chapter Two subsequent to the discovery that *CYP6P9* is a combination of *CYP6P13* and *CYP6P9* (Matambo *et al.,* 2010). The cDNA probe sequence of the *CYP6P3* gene (*CYP6P9* ortholog in *An. funestus*) on the detox chip was aligned to both the *CYP6P13* (Genbank accession number: EF152577) and *CYP6P9* (Genbank accession number: EU450763) gene sequences to determine the

relationship between the three sequences. The cDNA probe on the microarray slide had a 81% sequence identity to both *CYP6P9* and *CYP6P13*. Due to both sequences having high, similar percentage identities to the cDNA probe, primers for both *CYP6P9* and *CYP6P13* were used.

3.5.6.1 RNA isolation

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

3.5.6.2 cDNA synthesis

Total RNA was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, 4387406). Total RNA (2µg) was added to 10µl 2x RT buffer, 1µl 20x enzyme mix and nuclease-free water to give a reaction volume of 20µl. The reaction was incubated at 37ºC for one hour, 95ºC for five minutes and at 4ºC for 30 minutes. 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.

3.5.6.3 Primer design

Primer sequences for *CYP6P9* and *CYP6P13* were used from Chapter Two. Primers were designed for the *CYP6M7* and *COI* genes using the *An. funestus* gene sequences (www.ncbi.nlm.nih.gov/). These primers were designed using Beacon Designer 3.0 software (Bio-Rad, Hercules, CA, USA). The *rsp 7* (EF450776) primers were used as a reference gene to normalize data. Two different sets of primers were designed for the

CYP6M7 gene (Table 3.2). However, amplification of the product was unsuccessful after numerous attempts to optimize the cycling conditions, primer and cDNA concentrations.

Table 3.2 Primers used in 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	59.4/79.00	Primer set designed in this study
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'	* *	* *	Primer set designed in this study
CYP6P9	**Fwd** 5' AGA TGT GAT TGG CAC CTG T 3' **Rev** 5' TCG ATA TTC CAC CGT TTC CT 3'	232	55/ 82.00	Primer set designed in this study
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	Matambo, 2008
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 59.4ºC for *COI*, 55ºC for *CYP6P9* and 59.1ºC for *CYP6P13*.

3.5.6.4 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.

The cycling conditions for each primer set are presented in Table 3.2. Each biological sample was repeated three times on a plate. Three biological repeats were done on different days. All amplified products were sequenced in both directions to confirm the product. The data were analysed using the relative quantification method (Pfaffl, 2001).

All qPCR products were cloned into a vector due to the small fragment length of the product (as per methods section in Chapter Two). These cloned PCR products were sent to Macrogen for sequencing to verify that the correct gene was amplified in qPCR.

3.6 Results

Results from Chapter Two have shown that both females and males are resistant to permethrin, although mortality rates varied between the two sexes. Therefore, gene expression was determined separately in 3-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 hybridisation of the targets to the chip. The hybridisation temperature was reduced from 42ºC to 38ºC and lower stringency wash buffers and shorter washing times were used. The

slides were left in wash solution 1 for 2 ½ minutes instead of 5 minutes, wash solution 2 for 5 minutes instead of 10 minutes and the last wash in solution 3 for 1 minute instead of 2 minutes. These optimized conditions were similar to those used for cross-species hybridisation to *An. stephensi* (Vontas *et al.,* 2007). After optimization of the hybridisation conditions, over 90% of the probes were detected as opposed to 55% obtained when using the more stringent, original hybridisation conditions described by David *et al.* (2005) (Figure 3.3). The percentage of probes detected was obtained by manually counting the number of fluorescent spots on the slides after hybridisation. All technical and biological repeats produced comparable results with a correlation consensus of 0.89 for the females and 0.91 for the males. The correlation consensus value indicates how reproducible the data are between different arrays using the same sample. The closer the correlation value is to 1, the stronger the correlation between the samples.

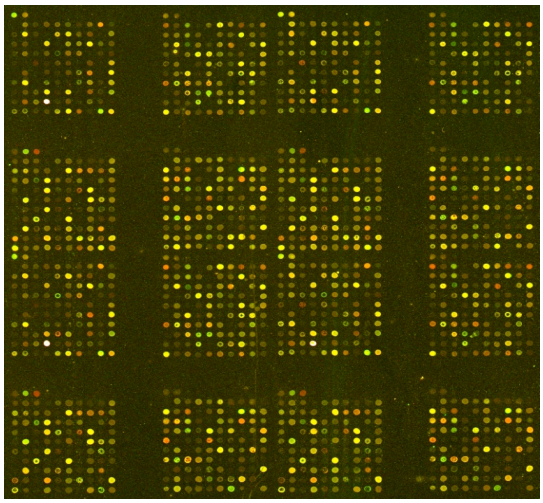

Figure 3.3 Microarray image generated by hybridising the *An. funetsus* targets onto an *An. gambiae* chip

3.6.1 Microarray data analysis

The microarray data were run in the Limma software package version 2.12.0, which was run in the program R version 2.8.0 (http://cran.r-project.org/bin/windows/base/old/2.8.0/). The data were displayed in various ways as a quality measure to detect any inconsistencies. An MA plot of the raw data for each array was generated using the command: [plotMA(RG,array=1,legend = T,ylim=c(-2,2))]. An MA plot compares the values of the green dye (Cy-3) against the red dye (Cy-5), where M is the intensity ratio and A is the average intensity for a dot in a plot. The plot contains information on the spike in controls, genes and other controls that are present on the array. Most of the non-differential genes and controls are expected to fall within the region of 0 and the differentially expressed genes fall either above or below 0. From the results obtained in this study, it was found that most of the differentially expressed genes and ratios fell either above or below zero. The calibration controls and non-differential genes fell in the region of zero as is expected in a MA plot (Figure 3.4).

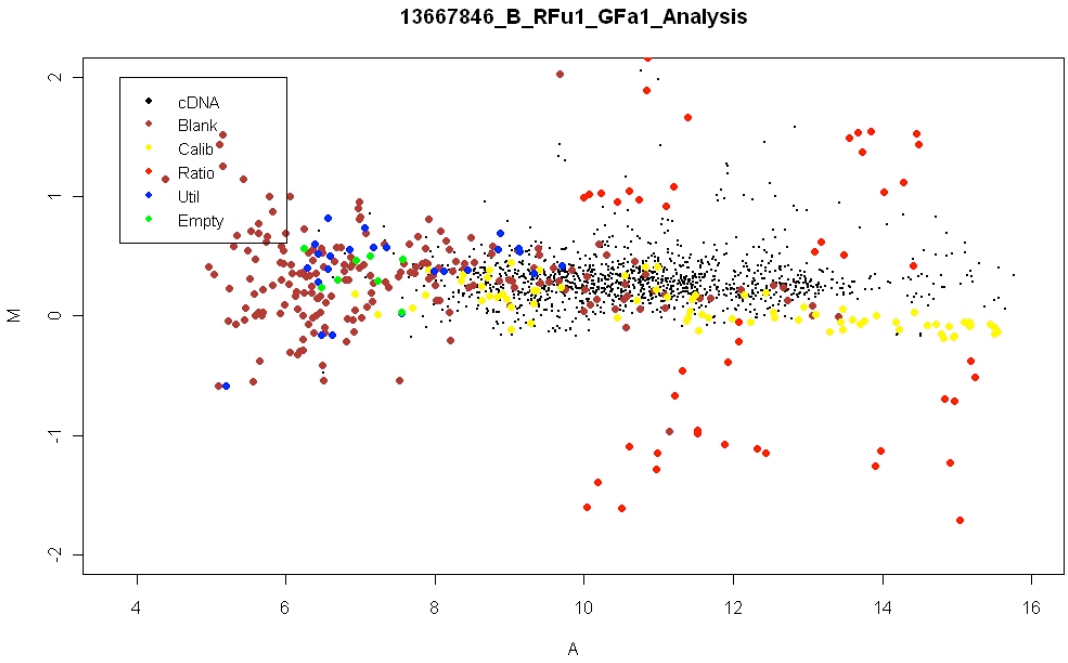

Figure 3.4 MA plot of a single array

The print layout of each slide was also examined. The print layout is the assembly of the spots on the arrays. Each array corresponds to a print tip on the print head used to print the arrays and the layout of the blocks on the array corresponds to the layout of the tips on the print head (Smyth *et al*., 2006). The print layout can be examined by using the command: [RG$printer][imageplot(log2(RG$Rb [,1]),RG$printer,low="white",high="red")]. This allows one to view the image in both the green and red fluorescence, hence determining any inconsistencies between dyes and slides. An example of the print layout obtained in this study (Figure 3.5) showed that there were no inconsistencies between the dye swap slides. This can be seen by the reciprocal image produced.

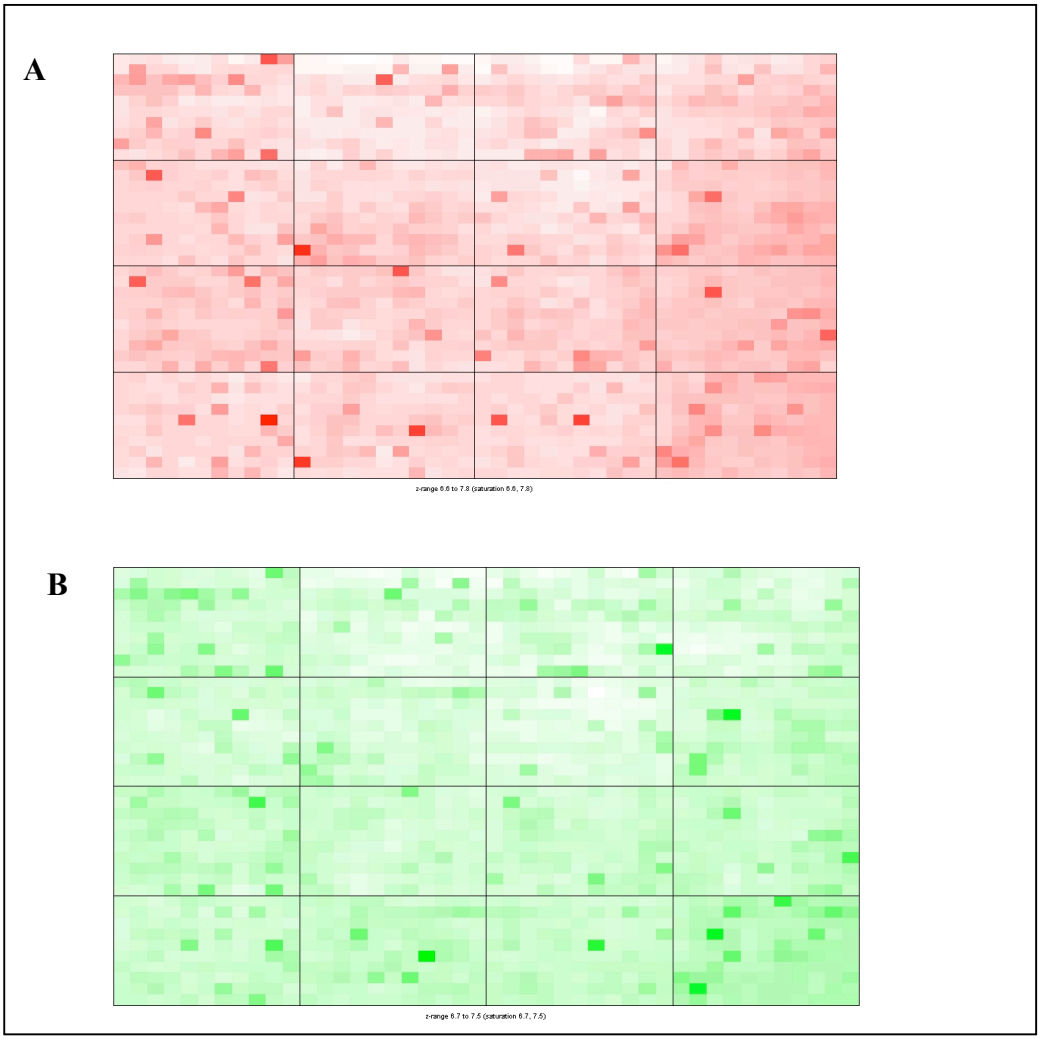

Figure 3.5 The print layout of a slide presented in the red channel (A) and the green channel (B)

Background correction was carried out for each slide (Figure 3.6). This entails subtracting the background intensity from the foreground intensity for each spot using the command: [RGb<-backgroundCorrect(RG,method="normexp",offset=50)]. This adjusts the foreground in relation to the background intensities and thus generates only positive adjusted intensities, thereby avoiding negative and zero corrected intensities. The spike in controls in this plot usually stands out from the other spots. The data presented here show that the spots are comparative to standard results.

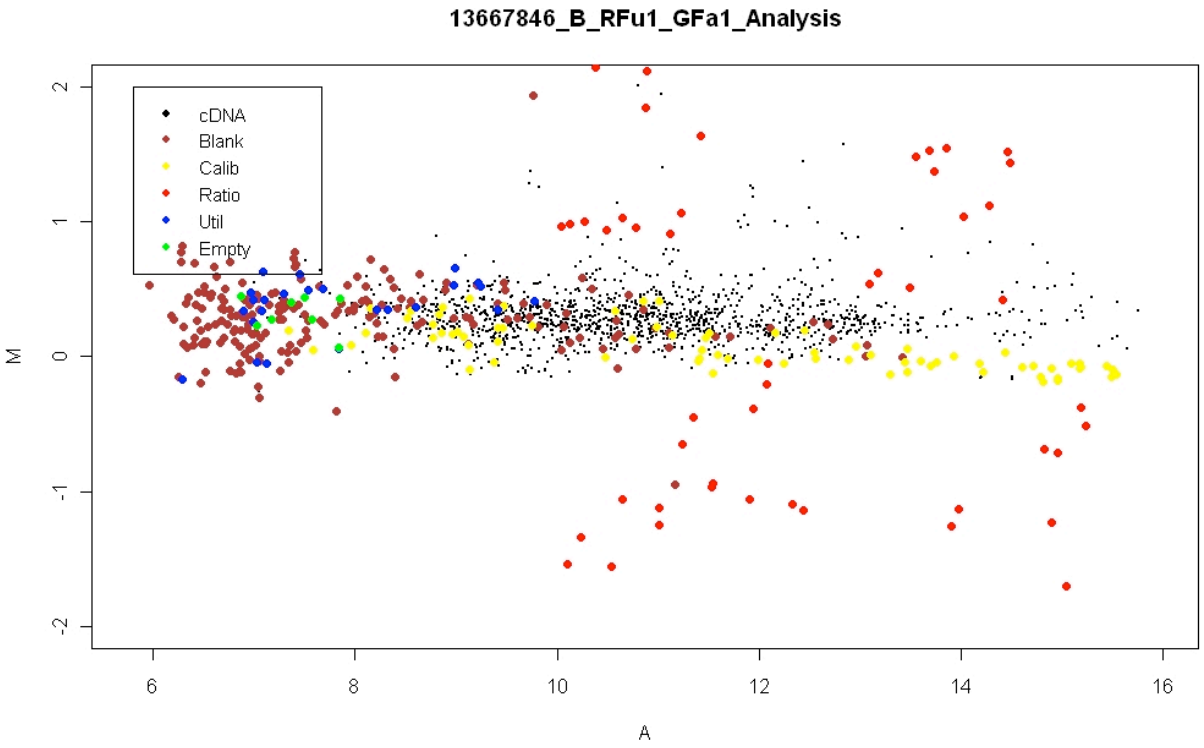

Figure 3.6 Graph presenting the background correction of a slide

Within array normalisation (Figure 3.7) was carried out. This method normalize the M values for each array separately [MA<normalizeWithinArrays (RGb,method= "control", controlspots=nonDE)]. The array normalisation data shown here are comparative to standard results.

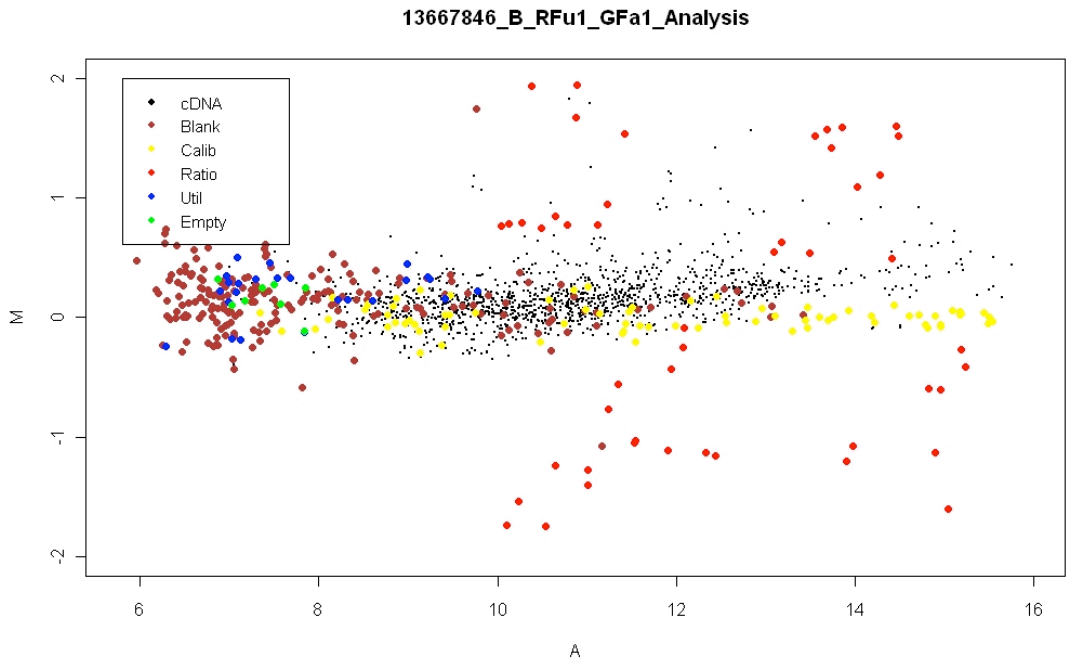

Figure 3.7 Graph presenting the within array normalisation of a slide

The plot densities within and between arrays were also examined using the following commands respectively, [plotDensities(MA[,1])] and [MAbet<-normalizeBetweenArrays(MA,method="Aquantile")]. Plot densities display the empirical densities for the individual green and red channels on the arrays. This has to be conducted to ensure that there are no significant variations between both channels and between arrays (Smyth *et al.,* 2006). The density plots produced in one example in this study showed that there was very little variation between the dyes (Figure 3.8) and arrays (Figure 3.9) and this can be seen by the consistent overlap of the red and green intensity values.

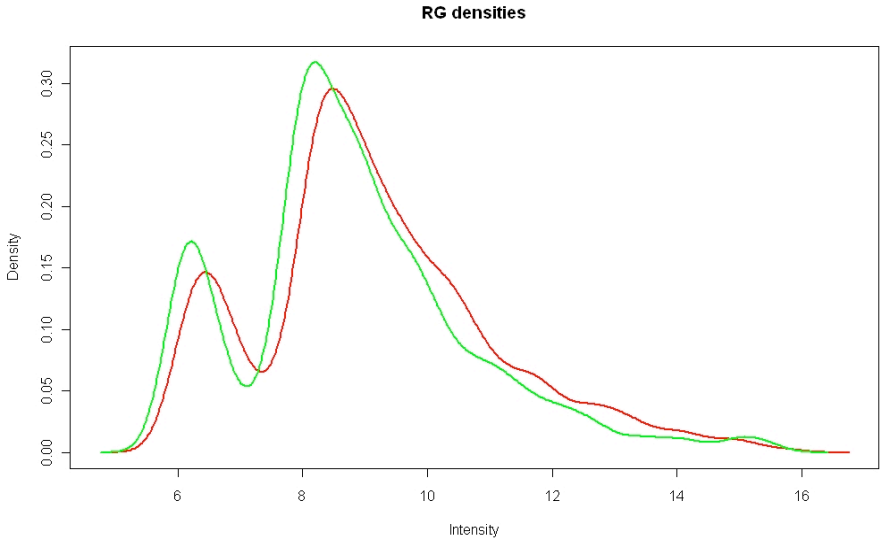

Figure 3.8 Presentation of the plot density within arrays

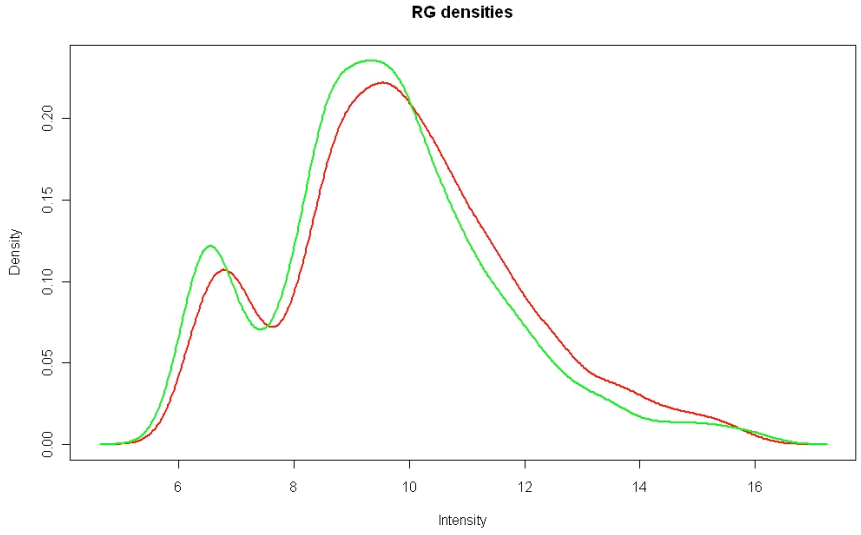

Figure 3.9 Plot density graph between arrays

3.6.2 Gene expression analysis

Genes were considered to be differentially expressed if they demonstrated a *B*-statistics value >2, ±1.5-fold change in expression between the two strains and a *P*-value of <0.001 over the entire experiment. No genes were found to be overexpressed in females and males from the susceptible *An. funestus,* FANG, strain. Out of a total of 255 gene probes on the *An. gambiae* detox chip, 1.18% of genes were significantly differentially expressed in the

resistant, FUMOZ-R, females as opposed to 9.45% in males, of the same strain. Three genes were highly expressed in the females of the resistant *An. funestus,* FUMOZ-R, strain (Figure 3.10). The gene with the highest fold change in expression (5.4-fold) in the females was cytochrome P450 gene *CYP6P9* (*CYP6P3* orthologous gene in *An. gambiae*) (Table 3.3). The cytochrome oxidase I gene, *COI*, was 2.7-fold overexpressed and the third gene, also a member of the cytochrome P450 enzyme family, *CYP6M7* (*CYP6M3* orthologous gene in *An. gambiae*) was 1.8-fold overexpressed.

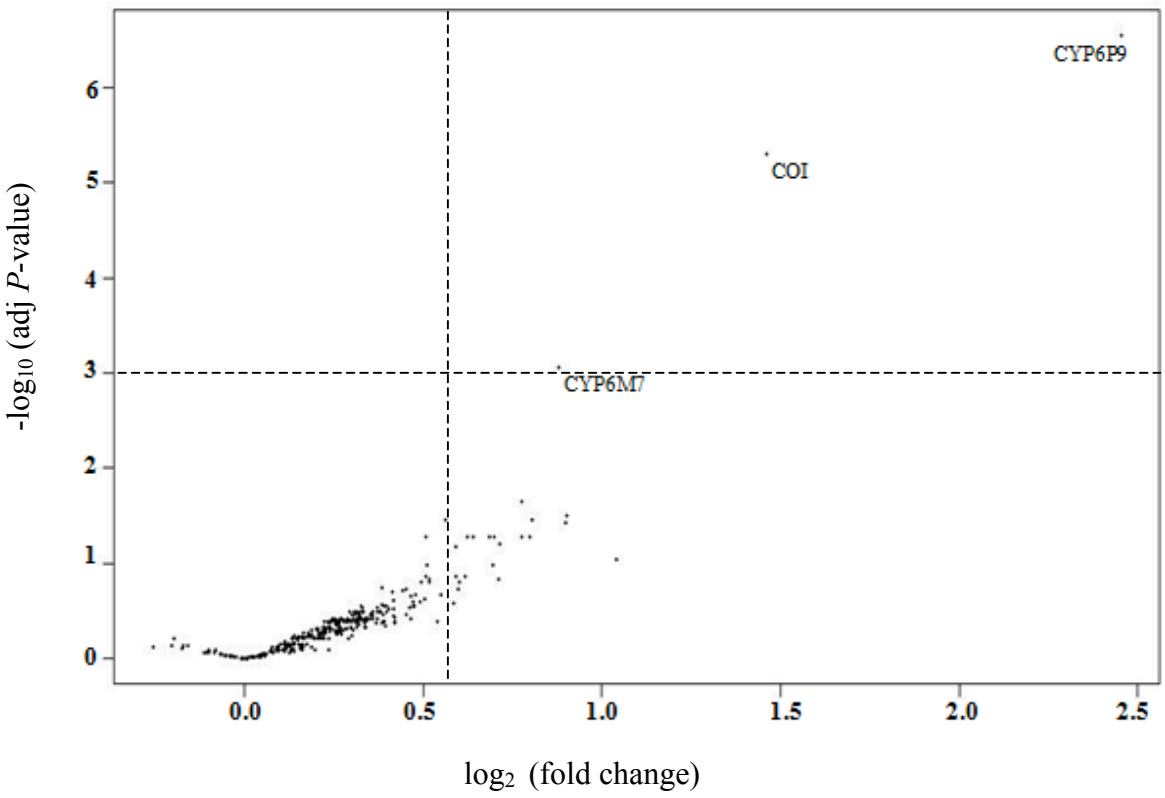

Figure 3.10 Volcano plot representing genes differentially expressed in females of the *An. funestus* strain, FUMOZ-R
The horizontal line represents the cut off for the level of significance α=0.001 and vertical lines indicate cut off for the 1.5-fold change threshold

A total of 24 genes were significantly differentially expressed in males from the resistant, FUMOZ-R, strain. Fifty percent of these genes (n=12) belonged to the cytochrome P450 enzyme group, 17% (n=4) to glutathione *S*-transferases, 8% (n=2) to Copper-Zinc (Cu-Zn)

superoxide dismutase, 8% (n=2) to the thioredoxin (TRX) group and the remaining 17% (n=4) to other groups (Figure 3.11). Three genes that were highly expressed and common to both sexes included the cytochrome P450 genes, *CYP6P9* (*CYP6P3* orthologous gene in *An. gambiae*) (6.0-fold), *CYP6M7* (*CYP6M3* orthologous gene in *An. gambiae*) (3.6-fold) and cytochrome oxidase I, *COI* (2.9-fold). Other differentially expressed genes in the males are shown in Table 3.3.

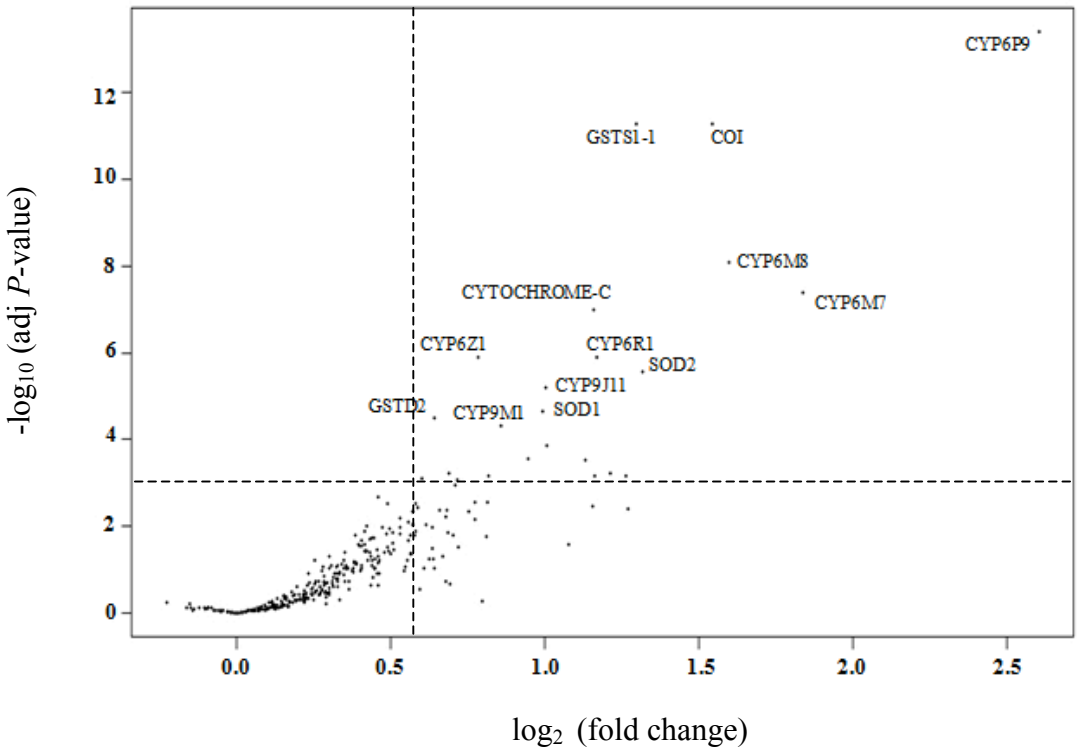

Figure 3.11 Volcano plot representing genes differentially expressed in males of the *An. funestus* strain, FUMOZ-R
The horizontal line represents the cut off for the level of significance α=0.001 and vertical lines indicate cut off for the 1.5-fold change threshold

Table 3.3 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	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*-transferases	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*-transferases	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*-transferases	AGAP009946	1.6	1.01×10^{-03}
CYP6M1 (CYP6M1)	Cytochrome P450	AGAP008209	1.6	5.96×10^{-04}
GSTD2	Glutathione *S*-transferases	AGAP004165	1.6	3.18×10^{-05}
CYP9J3 (CYP9J12)	Cytochrome P450	AGAP012291	1.5	7.71×10^{-04}

The remaining differentially expressed genes in males from the cytochrome P450 group were *CYP6M8* (*CYP6M2* orthologous gene in *An. gambiae*) (3.0-fold), *CYP6R1* (2.2-fold), *CYP12f2* (2.2-fold), *CYP6AG1* (2.0-fold), *CYP9J11* (*CYP9J5* orthologous gene in *An. gambiae*) (2.0-fold), *CYP9M1* (1.8-fold), *CYP6Z1* (1.7-fold), *CYP6AG2* (1.6-fold), *CYP6M1* (1.6-fold) and *CYP9J12* (*CYP9J3* orthologous gene in *An. gambiae*) (1.5-fold). Genes that were highly expressed in the males from the glutathione *S*-transferases (GST) group included *GSTS1-1* (2.5-fold), *GSTS1-2* (1.7-fold), *GSTMS3* (1.6-fold) and *GSTD2* (1.6-fold). Two genes from the Cu-Zn superoxide dismutase group were also overexpressed in the males, *SOD2* (2.5-fold) and *SOD1* (2.0-fold). Two genes from the thioredoxin group were overexpressed, *TRX1* (2.4-fold) and *TRX3* (2.2-fold). The remaining genes were *Actincytopl* (2.3-fold), *Cytochrome*_C (2.2-fold) and serine protease gene *SP8898* (1.9-fold).

Out of a total of 255 genes on the *An. gambiae* detox chip, 18 probes failed to produce any signal on the array (Figure 3.12).

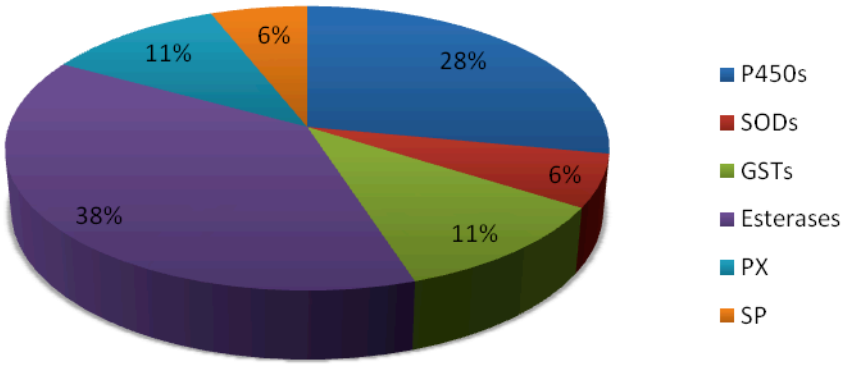

Figure 3.12 Percentage of genes that did not hybridise to the *An. gambiae* detox chip in both the females and males from FUMOZ-R resistant and the FANG susceptible strains

Five of these genes, *CYP306A1*, *CYP6P4*, *CYP4C28*, *CYP305A3*, and *CYP6Z2* belonged to the cytochrome P450 group (Table 3.4). Only two genes belonged to the GST group, *GSTD4* and *GSTD3* and the HPX (Peroxidases) group, *HPX7* and *HPX5*. 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 are available for *An. funestus* (Amenya *et al.,* 2005) 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 differentially expressed genes and one gene that did not produce any signal on the slide and these include: *COI, CYP6M1, CYP6M8, CYP9J11, CYP9J12, CYP6P9, CYP6P13, CYP6M7* and *CYP6P4* (Table 3.5). All genes had a percentage similarity >40%. The three genes with the highest percentage similarity were genes *CYP6P9* (81%), *CYP6P13* (81%) and *CYP6M1* (79.4%). Surprisingly, no signal was detected for *CYP6P4* despite the 75% sequence similarity between the two species.

Table 3.4 List of genes that did not hybridise onto the *An. gambiae* detox chip in both the females and males of the resistant and susceptible *An. funestus* strain, FUMOZ-R and FANG respectively

Gene Name	Group	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
HPX5	Peroxidase	AGAP000051
SP21408	Serine protease	AGAP004015

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 (Amenya *et al.,* 2005). The possibility that the microarray results are confounded by cross hybridisation of probes to multiple genes cannot be

excluded. Therefore, quantitative PCR was performed to validate the results from the genes found overexpressed in both sexes.

Table 3.5 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

3.6.3 Validation

Quantitative PCR (qPCR) was used to validate the microarray results for the three genes overexpressed in both sexes. The up-regulation of *CYP6P9, CYP6P13* and *COI* in the resistant FUMOZ-R strain in both females and males is presented in Figure 3.13A and Figure 3.13B, respectively. The females had a fold change of 67 for *CYP6P9,* eight for *CYP6P13* and four for the *COI* gene. The males had a fold change of 51 for the *CYP6P9* gene, 15 for *CYP6P13* and one for *COI* in the qPCR analysis.

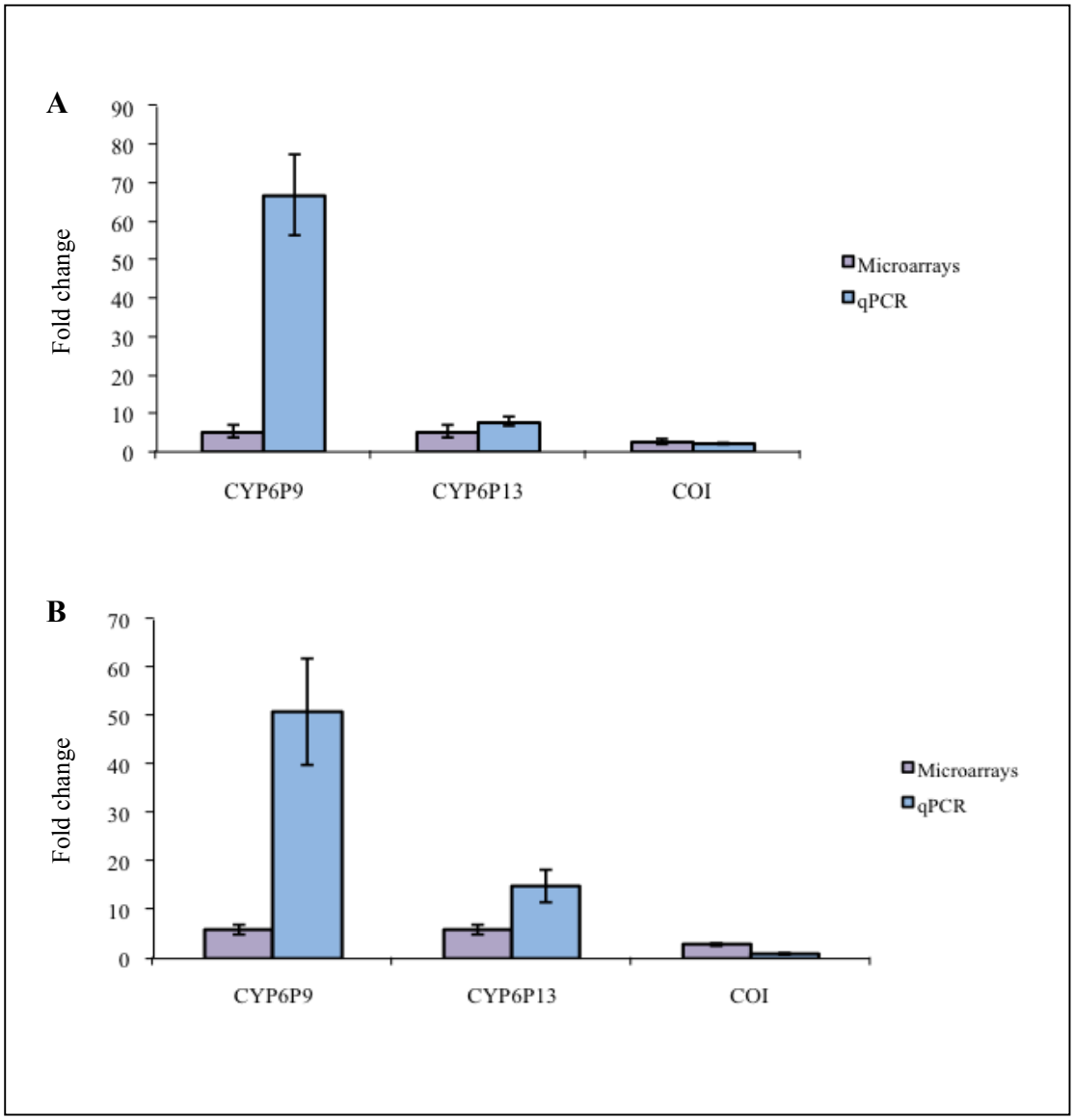

Figure 3.13 Comparison of qPCR and microarray results for *CYP6P9, CYP6P13* and *COI* genes in (A) females and (B) males

The comparison of all three genes for the qPCR and microarray data within each sex shows that only two genes, *CYP6P13* and *COI* were statistically significant in the females (Figure 3.13). However, each gene is overexpressed 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 hybridisation conditions and this could affect fold overexpression 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 (Amenya *et al.,* 2005) (Table 3.2). However, amplification of the product was unsuccessful for both primer pairs even after numerous attempts.

3.6.4 Sequencing

All amplified genes verified in the qPCR experiments were cloned into a vector (as per section 2.4.2.6) for sequencing by Macrogen (Korea). A BLAST search was run on the *COI* sequence and this gene had a 98% match to the *An. funestus* cytochrome oxidase subunit 1 (*COI*) gene, partial sequence (Accession number: AY423059.1). The sequence results for the *CYP6P9* and *CYP6P13* genes are presented in section 2.5.7.

3.7 Discussion

Gene expression in 3-day old *An. funestus* females and males was determined using the *An. gambiae* detox chip developed by David *et al.,* (2005). This study used cross-species (heterologous) hybridisation of *An. funestus* samples to a *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.* (2002) used human microarrays to study gene expression in pigs. They found that the reproducibility of microarray hybridisation of pig cDNA to human microarrays was high. Cross-species

hybridisation has also been conducted on rhesus macaque monkeys using a human high-density Affymetrix oligonucleotide array (Chismar *et al.,* 2002). Their results confirmed that a large number of genes were differentially expressed, thereby validating the use of cross-species hybridisations for nonhuman primate studies. Adjaye *et al.* (2004) used human and bovine fetal brain target samples on a human cDNA microarray. Highly 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* (Vontas *et al.,* 2007) and *An. arabiensis* (Müller *et al.* 2008a). This is the first study to use microarrays to determine the genetic basis of pyrethroid resistance in *An. funestus.*

The heterologous hybridisation approach was used successfully in this study and approximately 90% of the probes were detected. Three genes common to both sexes, were differentially expressed, *CYP6M7*, *CYP6P9* and *COI* whilst *CYP6P9, CYP6P13* and *COI* (although at higher levels when compared to microarray results) were confirmed to be overexpressed in association with pyrethroid resistance using qPCR analysis. The fact that *CYP6M7* could not be amplified from *An. funestus* mRNA could possibly imply that it is a pseudogene, but further studies are needed before such a conclusion is made. The lower hybridisation stringencies can result in hybridisation of another closely related P450 gene to the *CYP6M7* probe. This hypothesis can only be validated when a full genome sequence of *An. funestus* is available. Wondji *et al.* (2009) 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 *CYP6P9* gene has been implicated in insecticide resistance in *An. funestus* in various studies (Amenya *et al.,* 2008; Wondji *et al.,* 2009) prior to differentiation of this gene. Therefore, in this study, the *CYP6P9* and *CYP6P13* primers were designed in the regions that contained nucleotide base pair differences to differentiate between these two duplicate genes. This would ensure that the product obtained was the correct product. The fact that this gene (*CYP6P9*) was identified through a cross-species microarray holds promise for other similar studies.

Interestingly, the *CYP6P3* (*An. gambiae* ortholog) was also found to be differentially expressed (2.82-fold) in field caught *An. gambiae* adults from Ghana (Müller *et al.,* 2008b). The *CYP6P3* gene was also found at significantly high levels (2.6-fold) in *An. arabiensis* adults from Cameroon (Müller *et al.,* 2008a). This gene was also found to be overexpressed in pyrethroid resistant populations from Benin and Nigeria (Djouaka *et al.,* 2008).

In this study, the *COI* gene has been found to be differentially expressed in the pyrethroid resistant FUMOZ-R females. The *COI* gene was also found to be differentially expressed in an *An. arabiensis* resistant strain (Nardini, per comms). The mitochondrial cytochrome oxidase I (*COI*) gene has been used as a genetic marker in evolutionary studies and serves to transfer electrons and protons across the membrane (Lunt *et al.,* 1996). The abundance of the mitochondrial *COI* transcripts in the females from the resistant strain may play a role in the control of insecticide resistance and, therefore, resistance should also be studied at the mitochondrial level as well as at the nuclear stage. This might explain why the females were more resistant than the males (Chapter Two). However, functional genomic studies would be needed to confirm this. It should be noted that the *COI* gene was overexpressed

in both the females and males at almost the same level (females: 2.7 fold; males: 2.9 fold). However, in the qPCR analysis, the fold change differed between the two sexes (females: 4.0 fold; males: 1.0 fold). The results from the qPCR analysis however are more accurate due to the use of gene specific *An. funestus* primers as opposed to the probes on the detox chip, which are *An. gambiae* specific. Hence, the interpretation of the results is based on the qPCR results.

The observation that additional genes were up-regulated in males in the FUMOZ-R strain was surprising given that the resistance phenotype finds a stronger expression in females (Chapter Two). 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) (Chary *et al.,* 1994; Jamieson *et al.,* 1994; Park *et al.,* 2005), thioredoxins (TRX) (Bauer *et al.,* 2002) and glutathione *S*-transferases (GSTs) (Zou *et al.,* 2000; Singh *et al.,* 2001; Ding *et al.,* 2003). This suggests that male mosquitoes experience oxidative stress in a different manner to that of females.

A total of 12 cytochrome P450s were significantly differentially up-regulated in the males as opposed to only two in the females. This difference may be ascribed to other biological functions (such as pheromone production) that P450s are associated with in males. Cytochrome P450 enzymes are also involved in the metabolism of other xenobiotics (Bergé *et al.,* 1998; Scott and Wen, 2001). The role of these P450's in resistance needs to be clarified and it is unclear why there are additional up-regulated genes in the males, warranting further investigation.

Insect P450s also function in oxidative metabolism of insecticides. Monooxyegenases serve many functions but their most prominent role in Anopheles mosquitoes has been mediation of resistance to insecticides (Scott and Wen, 2001). Insect P450s consist of 6 CYP families, CYP4, 6, 9, 12, 18 and 28 (Bergé *et al.*, 1998; Hemingway and Ranson, 2000). The CYP4, CYP6 and CYP12 classes are found at elevated levels in insecticide resistant honeybees (Claudianos *et al.,* 2006), *An. funestus* (Amenya *et al.,* 2008) and houseflies (Tomita and Scott, 1995). The CYP6 class has been reported to play a role in insecticide resistance in *An. gambiae* (Nikou *et al.,* 2003; Müller *et al.,* 2007; Müller *et al.,* 2008b). The *CYP6M2* gene, which was found to be overexpressed in males in this study, was also found at high levels in studies by Djouaka *et al.* (2008) and Müller *et al.* (2008b). The CYP6 and CYP9 class featured among the genes up-regulated in males. The study conducted by David *et al.,* (2005) also found the *CYP6Z1*, *GSTS1*-2, *CYP612F2* and *CYP9J5* genes overexpressed in the *An. gambiae* strains. In a study conducted by Nikou *et al.* (2003), the *CYP6Z1* gene was found to be overexpressed in the *An. gambiae* strain.

The genes *SOD1* and *SOD2* have been found to be up-regulated in FUMOZ-R males. Superoxide dismutase (SOD) is active during defence against reactive oxygen species, which plays a role in cellular and DNA damage (Park *et al.,* 2005). For example, fungus lacking *SOD1* are more susceptible to oxygen and superoxide generating agents (Chary *et al.,* 1994; Jamieson *et al.,* 1994). It is thus suggested that FUMOZ-R males are more susceptible to oxidative stress than females explaining the differential expression of SOD genes in males. Duttaroy *et al.,* (2003) showed that a decreased level of the *SOD2* gene resulted in a reduction in the life span in *Drosophila. SOD2*, which is found in mitochondria, contains the bulk of detoxifying superoxide radicals. Therefore, the lack of

SOD2 activity will affect the life span of insects (Duttaroy *et al.*, 2003). The biological effect of the SOD genes on male longevity in FUMOZ-R will have to be investigated.

TRX1 and *TRX3* genes were differentially expressed in males. Thioredoxins play an important role in cellular function in insects such as cellular response to oxidative stress (Bauer *et al.,* 2002). The high levels of expression of the TRX genes in males in FUMOZ-R also suggests that males are more susceptible to oxidative stress when exposed to insecticides.

Four different glutathione *S*-transferases (GSTs) *GSTS1-1*, *GSTS1-2*, *GSTD2* and *GSTMS3* were up-regulated in the males. GST genes, like the SODs and TRX genes, have been implicated in protection against oxidative stress and the detoxification of endogenous and xenobiotic compounds (Zou *et al.,* 2000; Singh *et al.,* 2001; Ding *et al.,* 2003).

Only eighteen probes did not hybridise to the RNA target. A large portion of those probes (38%) belongs to the COE group with 28% from the P450 family. Surprisingly, two P450 genes, *CYP6P4* and *CYP6P1*, which were significantly differentially expressed in a study by Wondji *et al.,* (2009) did not hybridise to the array. The study conducted by Cuamba *et al.,* (2010) also failed to attain elevated levels of the *CYP6P4* gene in *An. funestus*. The *CYP6P1* and *CYP6P4* genes have been shown to have amino acid identity to that of *An. gambiae,* of 82% and 89% respectively on small partial sequences analysis (Amenya *et al.,* 2005). It is unknown why these genes did not hybridise to the slide. This will have to be investigated.

In addition to the 38% of carboxylesterases not hybridising to the slide, no carboxylesterases were found to be differentially expressed in females and males. High levels of esterases are known to mediate insecticide resistance in mosquitoes (Hemingway *et al.,* 2004). However, to date, no reports have implicated carboxylesterase genes in resistance in *An. funestus.*

The *An. gambiae* detox chip provided valuable information in terms of identifying potential genes involved in pyrethroid resistance in *An. funestus*. It is acknowledged that heterologous hybridisation is most likely to produce an under representation of differential expression of detoxifying genes, but it is currently the only tool available to screen multiple probes at once. This is a useful and an informative alternative given that the development of a species-specific *An. funestus* array will require full genome sequencing. This information will ultimately be used to understand the underlying mechanisms involved in pyrethroid resistance in this important malaria vector.

CHAPTER FOUR

Expression profile analysis of detoxification genes in different life stages of *Anopheles funestus*

4.1 Background

A mosquito's life cycle consists of four different phases. These include the egg stage, four larval stages, a pupa and an adult stage. The first three phases are aquatic and the adult phase is terrestrial. Such variation in habitat coincides with the development of a genome that accommodates a wide array of adaptive characteristics. Larvae and pupae in water are potentially exposed to chemical waste, algae and the toxic by-products of decomposed animal and plant matter all of which may affect their survival. Adults may be exposed to insecticides, blood contaminants from mammal hosts and other substances that could prove to be potentially harmful to them. Detoxification enzymes can provide protection against xenobiotic compounds in their natural environment. Previous studies have shown that an array of detoxification enzymes including cytochrome P450 monooxygenases (P450s), glutathione *S*-transferase (GSTs) and esterases are present at varying levels in different life stages in *An. gambiae* (Strode *et al.,* 2006). This suggests that these genes play different roles in the mosquito's life cycle. Variation in the expression of these enzymes through the developmental stages in *An. funestus* is currently undocumented.

4.2 Aims and Objectives

The aim of this study was to determine the transcription profile of the detoxification genes in the first instar (early aquatic stage), fourth instar (late aquatic stage) and 3-day old adult mosquitoes. The larval and adult stages of a mosquito are exposed to different environmental conditions e.g. diet and toxins. Quantifying differential expression of these genes at the different life stages will provide some insight into their role and function. As this study examined only the profile of detoxification genes in *An. funestus*, qPCR was not carried out. This will be investigated further in future studies.

4.3 Biological Material

4.3.1 Mosquito strains

The *An. funestus* pyrethroid resistant strain, FUMOZ-R, was used for this experiment. Cohorts of FUMOZ-R eggs were reared either to first or fourth instar larvae. All larvae were maintained on a diet of brewers yeast and ground dog biscuits. A mixture of 3-day old adult females and males (±8 adults each) were also collected.

4.4 Methods

RNA extractions were carried out for the adults (8 females and 8 males), first (±60) and fourth instar (±11) larvae. Microarray experiments were set up to allow for gene expression comparisons between first and fourth instar larvae, fourth instar larvae and adults and first instar larvae and adults. The indirect microarray design (Simon and Dobbin, 2003) was used to compare the expression profile of first instar larvae to that of fourth instar larvae. A

similar design was also used to compare expression profiles between fourth instar larvae and 3-day old adults (Figure 4.1). These data were then used to analyse differentially expressed genes between first instar larvae and adults without conducting additional experiments (Novoradovskaya *et al.,* 2004; Naidoo *et al.,* 2005). The procedures for the RNA extractions and microarray experiments can be found in Chapter Three. Details on data analysis are presented in section 3.5.5.



Figure 4.1. Design of microarray experiments using the indirect microarray design

4.5 Results

Hybridisation of the *An. funestus* targets resulted in over 90% of the probes being detected. Genes that displayed a *B*-statistics value >2, ±1.5-fold change in expression and a *P*-value of <0.001 in the statistical analysis were considered to be differentially expressed.

Six probes failed to produce any signal on the array when a comparison was carried out between the first and fourth instar larvae and these include *CYP305A4, SP21408, PX2, PX3, TRX2* and *Flightin.* Genes that failed to produce any signal on the array between the fourth instar and 3-day old adult comparison include *SP21408, PX2, PX3* and *TRX2.*The genes *PX2, PX3* and *TRX2*, however had hybridised on the slides used in the FUMOZ-R and FANG comparison. One reason for this occurrence could be due to printing errors.

4.5.1 First instar and fourth instar larvae comparison

Microarray comparisons were conducted between first and fourth instar larvae to determine which genes were being differentially expressed at these aquatic life stages. Out of 255 gene probes present on the *An. gambiae* detox chip, 76 genes (30%) were significantly differentially expressed, of which 67 showed increased gene transcription in the first instar larvae. Only nine genes were differentially expressed in the fourth instar larvae (Appendix B). Twenty two (33%) of the genes differentially expressed in the first instar larvae belonged to the P450 enzyme group.

Ten genes (15%) belonging to the esterase group, seven (10%) to GSTs, three (5%) to the serine protease (SP) genes, four (6%) to peroxidase (HPX) genes, three (4%) to the ABC transporter (ABCC) group, two (3%) to the NIT, thioredoxin peroxidise (TPX) and thioredoxin reductases (TRX) group and 12 (18%) to other groups, showed increase transcription (Figure 4.2). Information regarding the total number of gene groups present on the slide and the number of these genes within each gene family/class, differentially expressed on the slide is presented in Appendix B.

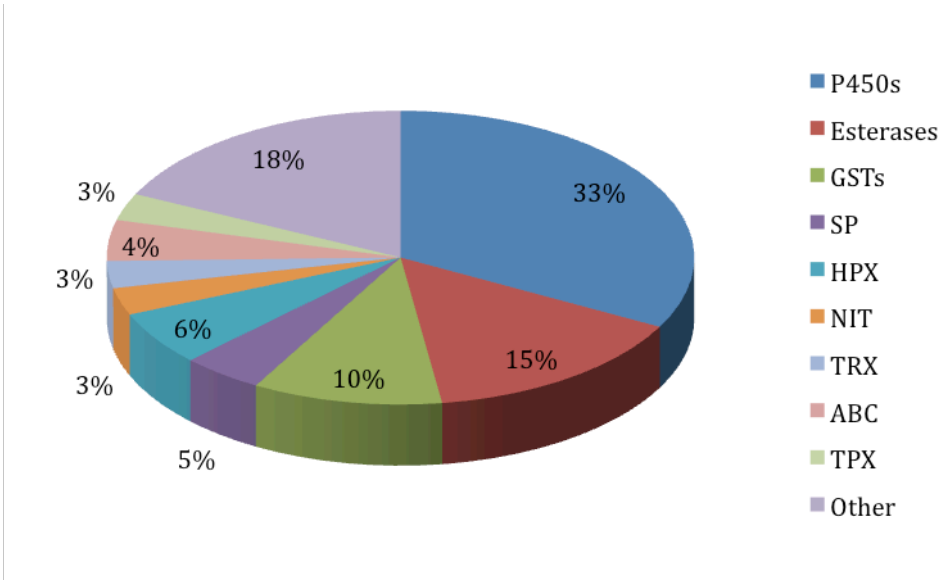

Figure 4.2 Proportions of genes differentially expressed by class in first instar larvae when compared to the fourth instar larvae

The top five genes differentially expressed in the first instar larvae include *COEAE1A* (5.5-fold), *CYP6M2* (4.6-fold), *ABCC11* (4.4-fold), *TubulinA* (4-fold) and *XD18014* (3.9-fold) (Figure 4.3).

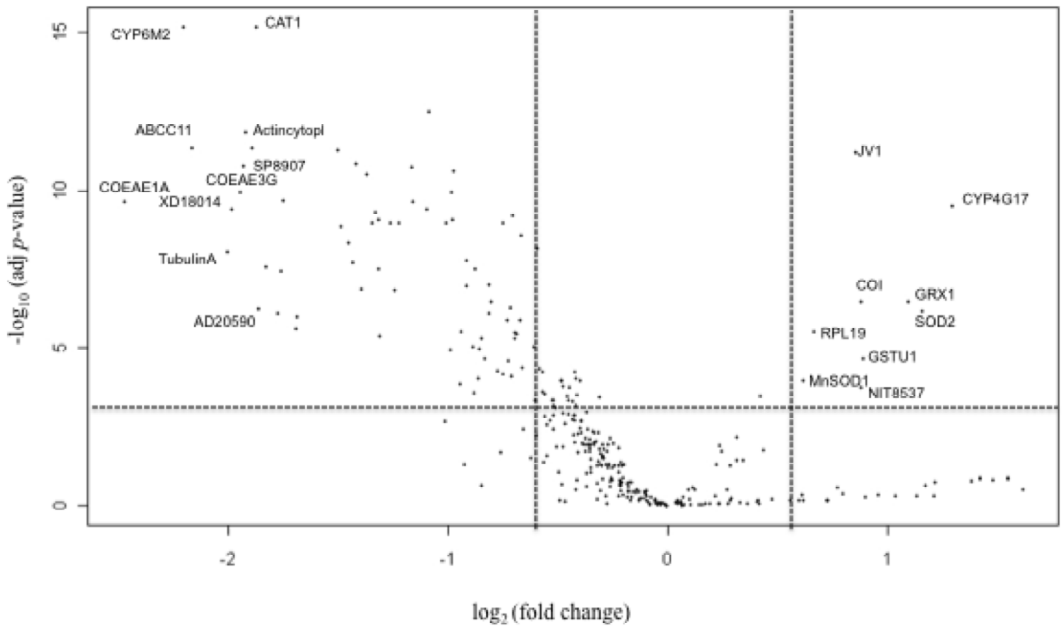

Figure 4.3 Volcano plot representing the top ten genes differentially expressed in the first and fourth instar larvae of the *An. funestus* strain, FUMOZ-R
The horizontal line represents the cut off for the level of significance α=0.001 and vertical lines indicate cut off for the two-fold change threshold

Genes differentially expressed in the fourth instar larvae include nine genes of which the most differentially expressed are *CYP4G17* (2.4-fold), *SOD2* (2.2-fold), *GRX1* (2.1-fold), *GSTU1* (1.8-fold), *NIT8537* (1.8-fold), *COI* (1.8-fold), *JV1* (1.8-fold), *RPL19* (1.5-fold) and Mn*SOD1* (1.5-fold) (Appendix B).

4.5.2 First instar larvae and 3-day old adult comparison

A comparison between first instar larvae and 3-day old adult mosquitoes revealed that 96 (38%) genes were differentially expressed out of a total of 255 genes on the slide. Ninety

one of these were differentially expressed in the first instar larvae (Appendix B) and the other five were differentially expressed in the adults.

A breakdown of those genes showing increased transcription levels in the first instar larvae revealed that the bulk (39%; n=35) were P450s. Analysis revealed that only 16 (18%) and 14 genes (15%) of the esterase and GST genes respectively were differentially expressed. The remaining differentially expressed genes in the first instar larvae include 4 (5%) belonging to the HPX group, 3 (3%) to the SP group, 2 (2%) belonged to the NIT, glutathione peroxidase (GP) and Tubulin groups and 13 (14%) belonged to other groups (Figure 4.4).

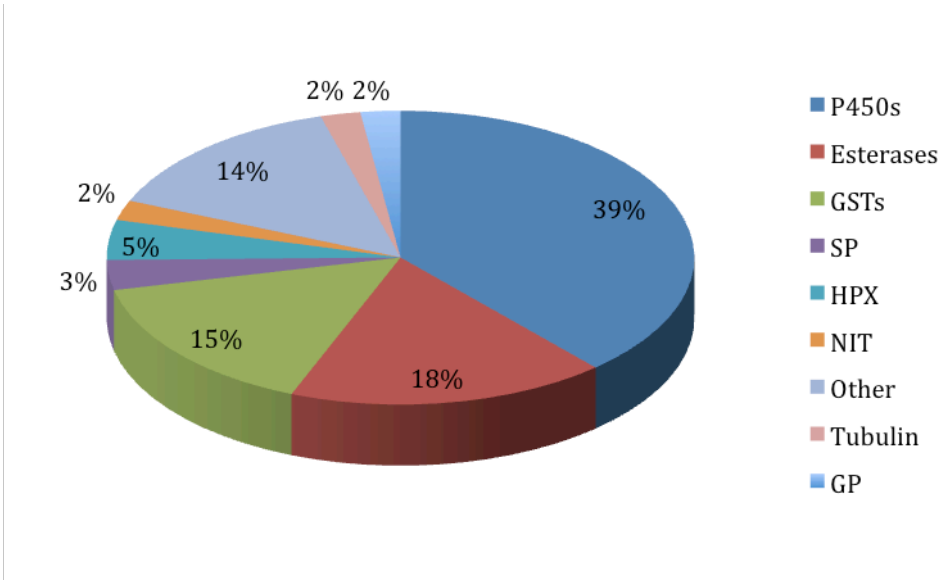

Figure 4.4 Proportions of genes differentially expressed by class in first instar larvae when compared to the 3-day old adults

The top five genes that were the most highly expressed in the first instar larvae were again *COEAE1A* (21.0-fold) and four other genes: *CYP4H24* (11.2-fold), *COEAE3G* (7.9-fold), *HPX15* (7.0-fold) and *TubulinA* (6.9-fold) (Appendix B).

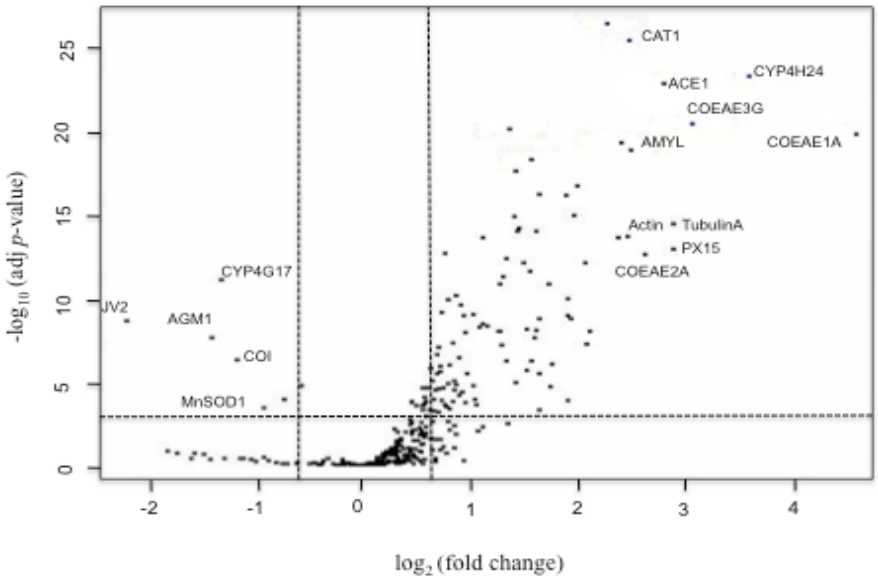

Figure 4.5 Volcano plot representing the top ten genes differentially expressed in first instar larvae and 3-day old adults of the *An. funestus* strain, FUMOZ-R
The horizontal line represents the cut off for the level of significance α=0.001 and vertical lines indicate cut off for the two-fold change threshold

Only five genes were differentially expressed in the adult mosquitoes (Figure 4.5): *JV2* (4.3-fold), *AGM1* (2.5-fold), *CYP4G17* (2.3-fold), *COI* (2.1-fold) and *MnSOD1* (1.6-fold) (Appendix B).

4.5.3 Comparison between fourth instar larvae and 3-day old adults

Fourth instar larvae and 3-day old adult mosquitoes were compared to each other to represent the change in detoxification genes of late aquatic stage vs. adults. Results showed that a total of 42 (17%) genes were differentially expressed on the slide, between these samples. Thirty five were differentially expressed in the fourth instar larvae (Appendix B) while seven genes were differentially expressed in the adult stage.

The differentially expressed genes in the fourth instar larvae include nine (25%) P450s, seven (20%) GSTs and six (17%) esterases. The remaining genes include two (6%) genes

belonging to the tubulin and the glutathione peroxidase (GP) group and nine (26%), belonging to other groups (Figure 4.6).

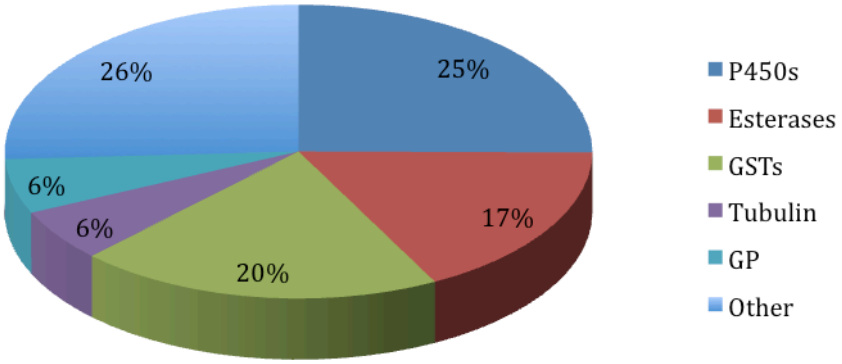

Figure 4.6 Proportions of genes differentially expressed in fourth instar larvae when compared to 3-day old adults

The top five genes which were most highly expressed in the fourth instar larvae include *CYP4H24* (4-fold), *CYP325D1* (3.9-fold), *TubulinB* (3.7-fold) and *GSTD10* (2.8-fold) (Figure 4.7). *COEAE1A* (3.9-fold) was also highly expressed as in the previous two analyses (section 4.5.1 and 4.5.2).

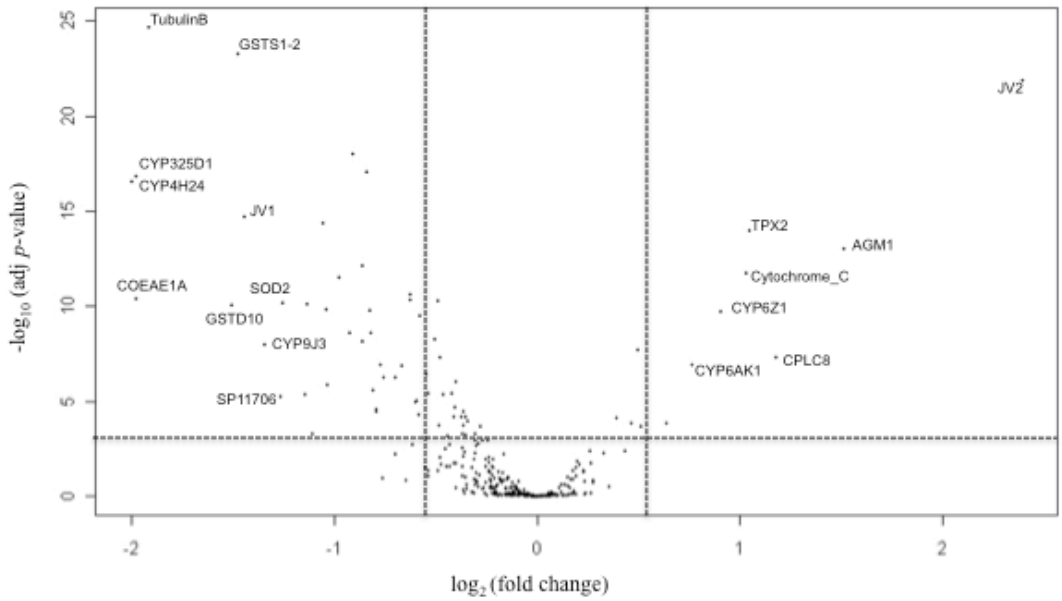

Figure 4.7 Volcano plot representing the top ten genes differentially expressed in fourth instar larvae and 3-day old adults of the *An. funestus* strain, FUMOZ-R
The horizontal line represents the cut off for the level of significance α=0.001 and vertical lines indicate cut off for the two-fold change threshold

In the adults seven genes were differentially expressed including two P450s and five other genes (Appendix B). Those showing highest differential expression were: *JV2* (5.2-fold), *AGM1* (2.8-fold), *CPLC8* (2.2-fold), *TPX2* (2.0-fold) and *Cytochrome_C* (2.0-fold) (Figure 4.7).

4.5.4 Genes expressed at specific life stages

The differential expression of certain genes was unique to a life stage, particularly in first instar larvae. These genes are listed in Table 4.1.

Table 4.1 Life stage specific genes*

First instar			Fourth instar	Adults
CYP301A1	COEAE2G	GPRNPY3	SOD2	AGM1
CYP307B1	COEAE3D	AMYL	JV1	JV2
CYP314A1	COEAE3G	CAT1	GSTU1	
CYP49A1	COEAE5G	Actincytopl	GRX1	
CYP4AA1	GSTD1-3	ABCC11	RPL19	
CYP4C26	GSTD1-6	XD18014	NIT8537	
CYP4C27	GSTD11	AD20590		
CYP4D16	GSTD6	NADPH_P450_red		
CYP4D22	GSTE1	TRXR		
CYP4H14	GSTU2	GSG8		
CYP4H18	GSTZ1	AP7862		
CYP4H19	SP8898	Xanthdehydro		
CYP4H24	SP8907			
CYP4J5	HPX15			
CYP6M2	HPX5B			
CYP6P2	HPX8			
CYP6P5	HPX9			
CYP9K1	NIT8503			
COE13O	NIT8730			
COEAE1A	ACE1			
COEAE2A	ACE2			
COEAE2D	TubulinA			

*Details on these genes are listed in supplementary tables provided in Appendix B

4.6 Discussion

This study used the indirect (reference) design approach to carry out the microarray experiment. The reference design method uses a common reference RNA sample (Naidoo *et al.,* 2005), in this case fourth instar larvae, which is compared to samples of interest i.e. first instar larvae and 3-day old adults. To account for dye effects, reverse-dye comparison was incorporated into the biological replicates. One of the reasons why this method was undertaken was due to the reduced number of microarray slides utilised and the reduced cost of reagents. In this study, 12 slides were used for hybridisations as opposed to 18 if a direct comparison between each sample was undertaken. Like most microarray designs, this method has its advantages and disadvantages. One of the disadvantages is that gene-specific dye bias can occur when using this method (Naidoo *et al.,* 2005). However, this can be overcome by performing reverse dye comparison, as was done in this study. One of the advantages of this method is that if a large quantity of reference sample is available, this design can be extended to accommodate a large number of sample comparisons (Naidoo *et al.,* 2005). An added advantage is the reduced cost of microarray reagents and number of slides used.

Gene expression profiles between the three different life stages of *An. funestus* (i.e. first and fourth instar larvae, first instar and 3-day old adults, and fourth instar larvae and 3-day old adults) were determined using the *An. gambiae* detox chip. A large number of genes in each comparison were differentially expressed. In addition, a large number of differentially expressed genes were common in all life stage. It is likely that the enhanced expression of these genes at particular life stages is important to each respective stage in terms of physiological regulation and development.

These results will provide some insight into the role and function of these genes. Interestingly, a larger number of genes were differentially expressed when comparing first instar (early aquatic stages) with either fourth instar larvae (late aquatic stage) or adults. Contrary to this the comparison between the late aquatic stage and adult stage revealed a relatively low number of genes being differentially expressed.

Most metabolic enzyme classes were found to be expressed at high levels during the larval stages, particularly in the first instar larvae. These gene groups include cytochrome P450 monooxygenase, esterase, glutathione *S*-transferase, ABC transporter, serine protease, NIT, thioredoxin peroxidase, peroxidase, tubulin and thioredoxin.

These data show that the P450s, esterases and GSTs are the most differentially expressed and it is interesting because these gene families are also involved in metabolising insecticides. The P450s differentially expressed in the larval stages belong to the CYP4, CYP6, CYP9 and other families, however, no genes from the remaining CYP12 gene family were differentially expressed. Cytochrome P450 monooxygenase is an important metabolic system that is involved in regulating compounds such as hormones, steroids, fatty acids and xenobiotics (Werck-Reichhart and Feyereisen, 2000; Scott and Wen, 2001; Liu *et al.,* 2006) and the metabolism of pheromones (Bergé *et al.,* 1998). However, other classes of P450 such as the *CYP314A1* (shade) gene, which belongs to the Halloween gene set, was differentially expressed in the first instar larvae. They have been found to play a role in embryonic development in *D. melanogaster* (Chavez *et al.,* 2000) and ecdysis and adult ovary development (Rewitz *et al.,* 2006; Strode *et al.,* 2006). This gene was also found to be expressed at high levels in larvae, pupae and adults in *An. gambiae* (Strode *et al.,* 2006). The biological function of this large enzyme family is extremely diverse,

complex and poorly understood. This study emphasizes the need to enhance and accelerate future studies in this field.

Many esterase transcripts were also differentially expressed in the larval stages. In contrast to this no esterase transcripts were found to be differentially expressed in adults. *COEAE1A* was the only esterase gene showing a high level of transcription in any of the larval comparisons (first vs. fourth and first vs. adult) and in the comparison between fourth instar larvae and adults. This implies that transcription of esterase genes is most important during the larval stages although their exact function is still unknown. Esterases are generally involved in the metabolism of lipids and fats in *Drosophila* larvae (Campbell *et al.,* 2003), the detoxification of xenobiotics (Russell *et al.,* 1996), detoxification of insecticides (Wheelock *et al*., 2008) as well as hydrolysis of the juvenile hormone and neurotransmitter acetylcholine (Taylor and Radic, 1994; Riddiford *et al.,* 2003).

Only a few esterases were differentially expressed that metabolise ß esterase substrates. However, a large number of esterases that metabolises α-naphthyl acetate type substrates were differentially expressed in most of the comparisons. This study however, did not aim to identify the function of the individual genes that were differentially expressed.

Various members of the GST enzyme family are associated with insecticide resistance (Grant, 1991; Grant *et al.,* 1991), metabolism and detoxification of endogenous and exogenous compounds (Ortelli *et al.,* 2003; Hemingway *et al.,* 2004), stress physiology, intracellular transport and numerous biosynthetic pathways (Wilce and Parker, 1994; Ding *et al.,* 2003). The group of GSTs that was the most differentially expressed in all life stage comparisons belonged to the delta group. Insect delta GSTs are known to play a key role in

detoxifying xenobiotics including insecticides and plant allelochemicals (Mittapalli, *et al.,* 2007).

All three of the above-mentioned enzyme groups have various biological functions and it is almost impossible to speculate on their individual roles in pyrethroid resistance in *An. funestus*. However, it is highly likely that these enzyme families are involved in biosynthesis pathways and detoxifying foreign compounds ingested by the aquatic stage larvae. These functions are shared with other gene families such as the ABC transporter genes and ACE genes. ABC transporter genes are thought to play a role in protecting mosquitoes from the toxic chemicals in their environment by metabolizing them (Roth *et al.,* 2003). ABC transporters act as a cells first line of defence, but do not function in the same way as detoxification mechanisms. Their functions are associated with transport processes and act by binding the ATP-binding domain and hydrolysing ATP to provide energy to transfer a range of molecules (e.g. toxic) across concentration gradients (Roth *et al.,* 2003).

Structural genes such as tubulin and cuticular genes were also differentially expressed. Tubulin, which was differentially expressed in the larvae, form part of the structural genes that contribute to the components of the cytoskeleton that mediate shape, motility, cell division and control of substances in the cell (Nielsen *et al.,* 2010). The *TubulinA* and *TubulinB* genes could be providing these functions in the larvae.

Immune genes such as serine protease were also differentially expressed. These genes are involved in immune protection against microbial agents. Other genes protecting against oxidative stress were also found to be differentially expressed in the larval stages and

included superoxide dismutases (*SOD*), peroxidases (*PX*), thioredoxin reductases (*TRX*), and thioredoxin peroxidases (*TPX*) (Zou *et al.,* 2000).

A number of genes were also exclusively highly differentiated in the adults. Two genes, *JV2* and *AGM1*, proved to be highly transcribed in the adults regardless of which larval stage they were compared to. The *AGM1* transcript is a midgut maltase, which is associated with sugar meal digestion (Ribeiro, 2003). As the adult mosquitoes were provided with a sugar solution it is not surprising that this gene was expressed in adult mosquitoes and not in the larvae. Based on this analysis, it is suggested that the cuticle gene, *JV2*, is adult specific. It is not unexpected to find a cuticle gene highly expressed as cuticle genes are essential for cuticle deposition in adults (Wood *et al.,* 2010). Studies conducted by Djouaka *et al.* (2008) and Awolola *et al.* (2009) also have found overexpression of cuticular genes in pyrethroid resistant *An. gambiae.*

CHAPTER FIVE

General Discussion and Conclusion

5.1 Discussion

The emergence of insecticide resistance in *An. funestus* has hampered both the control of this vector as well as transmission of the disease. Understanding the underlying mechanisms involved in insecticide resistance greatly facilitates our capabilities to develop novel or better insecticides.

Pyrethroid resistance in southern African *An. funestus* populations have been shown to be metabolic in nature and mostly due to increased monooxygenase activity (Hargreaves *et al.,* 2000; Brooke *et al.,* 2001; Casimiro *et al.,* 2006; Cuamba *et al.,* 2010). This study aimed to contribute additional information to this complex mechanism of pyrethroid resistance in *An. funestus*. This was firstly attained by determining the effect of age on pyrethroid resistance and examining the gene expression of two P450 genes; namely *CYP6P9* and *CYP6P13*, at different ages. The second was to identify changes in gene transcript levels associated with resistance by using the *An. gambiae* detox chip. The third and final aim in this study was to investigate how these detoxification gene transcripts change between three different life stages in *An. funestus*.

5.1.1 Effect of age on pyrethroid resistance and the gene expression of *CYP6P9* and *CYP6P13* at different ages

In the first part of this study, adults of different ages and of both sexes were exposed to 0.75% permethrin. It was found that in both sexes tolerance to permethrin decreased with

increasing age and reached its lowest point at day 14. The intake of a blood meal in females also did not have a significant effect on the percentage mortality when compared with unfed females of the same age. Female *An. funestus* can only become malaria infective when they reach an age of 10 to 14-days. The data from this study has shown that females older than 14-days old were more susceptible to insecticide than younger females. Reducing the vector population at this age and when the mosquito is older is imperative in any vector control intervention. The spraying of insecticides indoors will target and kill mosquitoes as soon as they enter houses for blood feeding and insecticide resistance interferes with the effectiveness of such an intervention. Pyrethroid resistance in *An. funestus,* from Mozambique, peaks when the risk to infection is also high which will in turn, enhance its ability to transmit the disease.

The gene expression of *CYP6P9, CYP6P13* and the combined effect of both genes were also determined by age and sex in a resistant strain of *An. funestus* mosquitoes. This was done to determine if these genes by themselves can explain the change in resistance observed over age. *CYP6P9* has previously been shown to be expressed at high levels in insecticide resistant *An. funestus* mosquitoes (Amenya *et al.,* 2008; Wondji *et al.,* 2009). However, these studies were done without separation of the individual duplicate genes, *CYP6P9* and *CYP6P13*. Therefore, the up-regulation of *CYP6P9, CYP6P13* and the combined effect of both genes was determined in 3, 5, 10, 14, 20 and 30-day old female and male pyrethroid resistant *An. funestus*. The variation in gene expression of the combined genes (*CYP6P9* and *CYP6P13*), and *CYP6P9* and *CYP6P13* individually, in FUMOZ-R females and males, was shown to vary with age. Also, the combined effect results were not comparative to the addition of the gene expression of the *CYP6P9* and *CYP6P13* genes, indicating that there may be other genes contributing to the effects seen.

This study is comparable to other studies conducted on age and gene expression. Work done by Lemos *et al.* (1996) showed that the expression of trypsin and aminopeptidase genes varied at different ages of *An. gambiae* mosquitoes. Marinotti *et al.* (2006) showed that variation of age and the intake of a blood meal in the same species resulted in the down-regulation in approximately 5% of all genes.

5.1.2 Changes in gene transcript levels in adult *An. funestus*

For the second part of the study, the transcript profile of detoxification genes in pyrethroid resistant *An. funestus* was determined using the *An. gambiae* detox chip. The comparison between the resistant FUMOZ-R and the susceptible FANG strain showed that no genes were differentially expressed in the susceptible mosquitoes for both sexes. In the resistant strain, only three genes were differentially expressed in the females, *CYP6P9* (5.4-fold), *COI* (2.7-fold) and *CYP6M7* (1.8-fold). In the males the same three genes were up-regulated, in addition to another 21 genes. Half of these belonged to the P450 group, which is implicated in insecticide resistance. This corresponds well with previous publications indicating that monooxygenases (P450s) are one of the main mechanisms (Hargreaves *et al.,* 2000; Brooke *et al.,* 2001; Casimiro *et al.,* 2006; Cuamba *et al.,* 2010). The top three differentially expressed genes for the females and males were validated using qPCR and these genes had a higher fold expression in the resistant strain than the susceptible. In this study, the use of the *An. gambiae* detox chip has provided valuable information regarding detoxification genes in *An. funestus*. It has also proven that the use of heterologous hybridisations can be useful in the initial screening of gene expression profiling in an organism. Although care should be taken in the interpretation of the results, verification of results is also essential to prevent false results.

5.1.3 Gene transcript changes in three life stages

Due to the hybridisation success of the *An. funestus* adult's cDNA to the *An. gambiae* detox chip, it was decided to further investigate the transcription profile at three different life stages of this species, within the resistant, FUMOZ-R, strain. The life stages studied were first and fourth instar larvae and 3-day old adults (females and males). In the microarray comparison between the life stages, the following results were obtained: 67 and nine genes were differentially expressed respectively in the first instar and fourth instar larvae comparison. A total of 91 genes were differentially expressed when the first instar larvae were compared to 3-day old adults. Very few (only five) genes were differentially expressed in the 3-day old adults in the same comparison. Comparison between the fourth instar larvae and 3-day old adults showed a total of 35 and eight genes differentially expressed, respectively. The results showed that between any two life stage comparisons, the common trend was that the early developmental phase of a mosquito always had significantly more genes differentially expressed as opposed to the late phase. The role and function of these individual proteins in larval life stages would provide insight into this phenomenon. As this microarray contains a large number of P450s, esterases and GSTs, it was not surprising to find numerous up-regulated genes within these enzyme families. Interestingly, no esterases where up-regulated in the adults, although elevated levels of esterases in the male accessory glands of *An. funestus* have previously been described (Green, 1977). The discrepancy in the two studies may be attributed to the following. In this study, the adult RNA used on the detox chip was obtained from both females and males, and not males only. Further, the up-regulation of esterases was not detected possibly because of the sequence differences between those on the chip, which belonged to *An. gambiae* and those hybridised to the slide, which belonged to *An. funestus.*

5.2 Further Investigations

A number of additional research questions were raised during this study, however, as the objectives in this study were well defined and time was a limiting factor, it was not possible to add these to the current project. Additional research on insecticide resistance in *An. funestus* would greatly benefit the understanding of these genes and their role in resistance. One way in which this could be done would be to test field populations from different localities in Africa and determine if there is a difference in the resistance mechanisms between field and laboratory reared populations of *An. funestus.* The transcription profile of the field population could also be established. These types of studies have recently been initiated.

Further research would also involve verifying, using qPCR, the remaining genes that were differentially expressed in 3-day old resistant males in the microarray experiment. As only three genes were up-regulated in the females and these were already verified using qPCR, only the genes up-regulated in the males need to be investigated. Only three genes were verified using qPCR in the present study due to time constraints. Further investigations will involve researching the roles these genes play in insecticide resistance in both females and males.

The use of cross-species microarrays provides a useful method for initial screening of up-regulated genes. However, the construction of a species-specific microarray platform for *An. funestus* would provide more accurate results. The sequencing of the *An. funestus* genome and the development of a *An. funestus* microarray will greatly aid in answering a large number of questions still unknown in this malaria vector.

It is important to note that microarray technology only observes changes due to gene expression; therefore, if a gene is associated with resistance due to point mutations, they will not be detected. Combining microarray and SNP analysis or QTL mapping would yield more thorough results in a larger scale project.

Another approach to studying insecticide resistance in this species would involve using gene knockdown RNAi technology. This could be used to target genes of interest (e.g. *CYP6P9*) by inhibiting gene expression at the translation stage or blocking the transcription of this gene. In the case of *CYP6P9*, this could result in the pyrethroid resistant strain, FUMOZ-R, becoming susceptible which will clarify the role of *CYP6P9* in pyrethroid resistance.

5.3 Concluding Remarks

To date various studies have been conducted on genes and gene expression in *An. funestus* (Amenya *et al.,* 2005; Amenya *et al.,* 2008; Wondji *et al.,* 2007; Wondji *et al.,* 2009; Serazin *et al.,* 2009; Cuamba *et al.,* 2010; Matambo *et al.,* 2010). Most of these have focused on only a few genes, and no research has been done on their expression profile in *An. funestus.* The present study has shown that various genes are up-regulated in association with resistance, but may not necessarily be involved in insecticide resistance. A large number of P450s and other genes could also be involved. Overall, this study has contributed to the understanding of *An. funestus* genes associated with insecticide resistance.

APPENDIX A

Recipes agar plates and broth

1. Luria Bertani Broth (1 litre)

   10g Bacto-tryptone

   5g Bacto-Yeast extract

   10g NaCl

   Adjust to 1L with distilled water and autoclave.

2. Luria Bertani Agar (1 litre)

   10g Bacto-tryptone

   5g Bacto-Yeast extract

   10g NaCl

   15g Bacto-Agar

   Adjust to 1L with distilled water and autoclave.

   Add Ampicillin (100 mg/ml), when LB agar is cool.

   Pour to plates when cooled.

3. Ampicillin (100mg/ml)

   1g ampicillin

   10ml deionised water

   Add ampicillin to water.

   Aliquot into 1ml tubes and store at -20°C until further use.

4. IPTG

   0.12g IPTG

   5 ml distilled water

   Mix and store at 4°C for many months.

5. Positive transformation Control

   The concentration of the plasmid is (1ng/µl).

   Dilute the positive plasmid 1:100 in dH_2O and use 1 µl for transformation.

APPENDIX B

Table 1 List of genes differentially expressed in first instar larvae in comparison to fourth instar larvae

Gene Name	Vectorbase Accession Number	Fold Change	Adj. *P*-value	Gene families/class numbers present on slide	Number of genes within families/class differentially expressed *
Cytochrome P450 monooxygenase					
CYP301A1	AGAP006082	2.83	5.42E-12	CYP3 (30)	4 (13%)
CYP307B1	AGAP008682	2.49	3.15E-08		
CYP314A1	AGAP002429	2.40	1.13E-09		
CYP325C2	AGAP002205	1.62	5.05E-06		
CYP49A1	AGAP005774	2.59	3.18E-11	CYP4 (30)	12 (40%)
CYP4AA1	AGAP003608	2.67	1.39E-11		
CYP4C26	AGAP000192	1.81	1.13E-05		
CYP4C27	AGAP009246	1.58	4.21E-05		
CYP4D15	AY062193 (GB)	1.71	5.19E-05		
CYP4D16	AGAP002418	1.64	7.85E-05		
CYP4D22	AGAP002419	1.75	3.30E-07		

CYP4H14	AGAP001861	1.92	3.01E-06		
CYP4H18	AY062195 (GB)	2.74	4.75E-09		
CYP4H19	AGAP000088	2.01	1.11E-09		
CYP4H24	AGAP000088	2.80	1.39E-09		
CYP4J5	AGAP006048	1.76	1.04E-07		
CYP6M2	AGAP008212	4.60	6.40E-16	CYP6 (29)	4 (13%)
CYP6P2	AGAP002869	1.97	9.04E-10		
CYP6P5	AGAP002866	2.63	1.35E-07		
CYP6Z1	AGAP008219	1.96	2.52E-11		
CYP9J4	AGAP012292	3.22	9.96E-07	CYP9 (9)	2 (22%)
CYP9K1	AGAP000818	1.93	0.000143		
Esterase					
COE13O	AGAP011507	1.65	2.66E-05	COE1 (2)	1 (50%)
COEAE1A	AGAP001722	5.55	2.28E-10	COEAE (16)	7 (43%)
COEAE2A	AGAP001723	3.38	3.65E-08		
COEAE2D	AGAP005757	2.34	1.11E-09		
COEAE2G	AGAP006723	1.59	1.31E-06		
COEAE3D	AGAP005758	2.70	1.88E-08		
COEAE3G	AGAP006724	3.85	1.10E-10		

COEAE5G	AGAP006726	1.63	6.44E-10		
ACE1	AGAP001356	1.82	9.46E-05	ACE (2)	2 (100%)
ACE2	AGAP000466	1.59	2.64E-09		
Glutathione S-transferase					
GSTD1-3	AGAP004164	1.98	1.08E-10	GSTD (15)	4 (26%)
GSTD1-6	AGAP004164	2.12	3.18E-13		
GSTD11	AGAP004378	1.89	1.70E-08		
GSTD6	AGAP004379	1.51	7.12E-09		
GSTE1	AGAP009195	1.85	9.42E-06	GSTE (8)	1 (12%)
GSTU2	AGAP003257	1.89	1.08E-07	GSTU (3)	1 (33%)
GSTZ1	AGAP002898	1.50	4.39E-05	GSTZ (1)	1 (100%)
ABC Transporter					
ABCC10	ENSANGG00000004998	1.68	6.71E-05	ABCCC (5)	3 (60%)
ABCC11	AGAP008436	4.49	4.72E-12		
ABCC12	AGAP007917	2.36	1.55E-07		

Serine Protease					
SP8898	AGAP003642	3.55	2.58E-08	SP (9)	3 (33%)
SP8905	AGAP003640	1.62	3.29E-06		
SP8907	AGAP003641	3.81	1.65E-11		
NIT					
NIT8503	AGAP012801	1.78	2.24E-05	NIT (4)	2 (50%)
NIT8730	AGAP012379	1.66	1.33E-06		
Thioredoxin peroxidase					
TPX2	AGAP011054	2.51	5.06E-10	TPX (5)	2 (40%)
TPX3	AGAP007543	1.80	5.12E-06		
Peroxidase					
HPX15	AGAP010810	3.42	7.89E-07	HPX (19)	4 (21%)
HPX5B	AGAP000051	2.23	2.28E-10		
HPX8	AGAP004038	1.84	3.15E-08		
HPX9	AGAP010899	2.54	1.13E-09		

Thioredoxin					
TRX1	AGAP009584	1.76	7.89E-07	TRX (4)	2 (50%)
TRXR	AGAP000565	2.48	4.12E-06		
Other					
TubulinA	AGAP001219	4.02	8.42E-09	Tubulin (2)	1 (50%)
XD18014	AGAP006220	3.96	4.01E-10	XD (3)	1 (33%)
Actincytopl	TC48694 (TIGR)	3.79	1.42E-12	Actin (2)	1 (50%)
CAT1	AGAP004904	3.66	6.40E-16	CAT (1)	1 (100%)
AD20590	AGAP010499	3.63	5.74E-07	AD (2)	1 (50%)
AMYL	AGAP002317	3.36	2.05E-10	AMYL (1)	1 (100%)
NADPH_P450_red	AGAP000500	3.23	2.45E-06	NADPH_P450_red (1)	1 (100%)
GPRNPY3	AGAP012378	2.49	8.44E-10	GPRN (7)	1 (14%)
Cytochrome_C	AGAP009537	2.14	4.01E-10	Cytochrome (2)	1 (50%)
GSG8	AGAP010647	1.98	1.22E-05	GSG8 (1)	1 (100%)
Xanthdehydro	4A3A-AAK-G-02-R (EMBL)	1.68	1.11E-09	Xanthdehydro (1)	1 (100%)
AP7862	AGAP007809	1.64	5.29E-07	AP (1)	1 (100%)

* These values represent the number of gene families/class within each group differentially expressed. These values are also represented as a percentage of the total number of that class present on the slide.

Table 2 List of genes differentially expressed in the fourth instar in comparison to first instar larvae

Gene Name	Vectorbase Accession Number	Fold Change	Adj. *P*-value	Gene families/class numbers present on slide	Number of genes within families/class differentially expressed *
Superoxide dismutase					
MnSOD1	AGAP010517	1.53	1.08E-04	SOD (4)	2 (50%)
SOD2	AGAP005234	2.23	6.99E-07		
COI	DQ465331 (GB)	1.84	3.30E-07	COI (1)	1 (100%)
CYP4G17	AGAP000877	2.45	3.13E-10	CYP4 (30)	1 (3%)
GRX1	AGAP011107	2.13	3.30E-07	GRX (3)	1 (33%)
GSTU1	AGAP000947	1.85	2.24E-05	GSTU (3)	1 (33%)
JV1		1.80	6.39E-12	JV (2)	1 (50%)
NIT8537	AGAP003515	1.84	1.75E-04	NIT (4)	1 (25%)
RPL19	AGAP004422	1.58	3.20E-06	RPL (1)	1 (100%)

* These values represent the number of gene families/class within each group differentially expressed. These values are also represented as a percentage of the total number of that class present on the slide.

Table 3 List of genes differentially expressed in first instar larvae in comparison to 3-day old adults

Gene Name	Vectorbase Accession Number	Fold Change	Adj. *P*-value	Gene families/class numbers present on slide	Number of genes within families/class differentially expressed *
Cytochrome P450 monooxygenase					
CYP301A1	AGAP006082	3.85	1.25E-17	CYP3 (30)	6 (20%)
CYP307B1	AGAP008682	2.90	3.09E-07		
CYP314A1	AGAP002429	2.65	6.03E-15		
CYP325A2	ENSANGESTG00000005673 (ETI ID)	1.85	4.33E-05		
CYP325D1	AGAP002206	3.24	7.47E-12		
CYP325G1	AGAP002196	1.66	4.96E-10		
CYP49A1	AGAP005774	3.00	3.88E-09	CYP4 (30)	15 (50%)
CYP4AA1	AGAP003608	3.05	4.52E-17		
CYP4C26	AGAP000192	1.89	2.58E-05		
CYP4C27	AGAP009246	1.85	1.92E-07		
CYP4C28	AGAP010414	2.88	1.41E-12		
CYP4C36	AGAP009241	3.04	8.63E-10		
CYP4D16	AGAP002418	1.77	7.32E-05		

CYP4D22	AGAP002419	2.01	9.19E-06		
CYP4H14	AGAP001861	2.40	2.89E-08		
CYP4H18	AY062195 (GB)	3.62	6.87E-05		
CYP4H19	AGAP000088	2.49	2.19E-13		
CYP4H24	AGAP000088	11.21	8.13E-24		
CYP4H25	AGAP001864	1.66	8.01E-05		
CYP4J5	AGAP006048	1.79	1.62E-05		
CYP4J9	AGAP006047	1.78	2.42E-08		
CYP6AA2	AY745221 (GB)	1.85	3.25E-05	CYP6 (29)	10 (34%)
CYP6AH1	AGAP007480	1.68	1.14E-05		
CYP6M2	AGAP008212	4.63	9.82E-27		
CYP6M3	AGAP008213	1.61	1.02E-07		
CYP6N1	AY028786 (GB)	1.62	1.46E-06		
CYP6P2	AGAP002869	1.90	5.72E-10		
CYP6P4	AGAP002867	2.61	6.87E-06		
CYP6P5	AGAP002866	2.82	1.01E-06		
CYP6Y1	AGAP008208	2.49	2.59E-07		
CYP6Y2	AGAP008207	1.63	3.80E-08		
CYP9J3	AGAP012291	2.05	0.000122	CYP9 (9)	4 (44%)
CYP9J5	AGAP012296	2.53	6.66E-21		

CYP9K1	AGAP000818	2.39	4.14E-09		
CYP9L1	AGAP012295	1.80	8.08E-06		
Esterase					
COE13O	AGAP011507	1.72	8.94E-06	COE1 (2)	1 (50%)
COEAE1A	AGAP001722	21.96	1.83E-20	COEAE (16)	10 (62%)
COEAE1F	AGAP006227	2.36	4.76E-09		
COEAE2A	AGAP001723	5.87	1.59E-13		
COEAE2D	AGAP005757	2.75	4.15E-13		
COEAE2G	AGAP006723	1.74	5.01E-07		
COEAE3D	AGAP005758	3.70	8.63E-10		
COEAE3G	AGAP006724	7.92	5.10E-21		
COEAE5G	AGAP006726	1.86	1.34E-10		
COEAE5O	AGAP002391	1.70	0.000116		
COEAE6O	AGAP002863	1.79	4.32E-06		
COEBE2C	AGAP005371	4.07	2.69E-08	COEB (4)	2 (50%)
COEBE3C	AGAP005372	1.59	1.77E-05		
COEJHE2E	AGAP005834	1.63	1.25E-06	COEJ (5)	1 (20%)
ACE1	AGAP001356	6.60	1.90E-23	ACE (2)	2 (100%)
ACE2	AGAP000466	2.13	1.25E-14		

Glutathione S-transferase					
GSTD1-3	AGAP004164	3.59	4.52E-17	GSTD (15)	8 (53%)
GSTD1-6	AGAP004164	2.63	1.55E-18		
GSTD10	AGAP004383	2.95	1.12E-08		
GSTD11	AGAP004378	2.91	3.40E-19		
GSTD3	AGAP004382	2.02	5.46E-10		
GSTD6	AGAP004379	1.69	1.36E-13		
GSTD7	AGAP004163	1.51	1.41E-05		
GSTD8	AGAP004171	1.56	4.32E-06		
GSTE1	AGAP009195	2.38	6.56E-12	GSTE (8)	2 (25%)
GSTE5	AGAP009192	1.73	6.42E-11		
GSTMS3	AGAP009946	1.62	1.44E-05	GSTM (3)	1 (33%)
GSTS1-2	AF513639 (GB)	2.67	4.20E-15	GSTS (2)	1 (50%)
GSTU2	AGAP003257	2.44	2.60E-12	GSTU (3)	1 (33%)
GSTZ1	AGAP002898	1.92	7.41E-09	GSTZ (1)	1 (100%)
Serine Protease					
SP11706	AGAP004014	3.28	4.94E-07	SP (9)	3 (33%)
SP8898	AGAP003642	4.98	1.72E-14		
SP8907	AGAP003641	4.19	4.45E-09		

Peroxidase					
HPX15	AGAP010810	7.00	7.15E-14	HPX (19)	4 (21%)
HPX5B	AGAP000051	3.25	9.67E-06		
HPX8	AGAP004038	2.14	2.00E-09		
HPX9	AGAP010899	3.06	1.39E-06		
NIT					
NIT8503	AGAP012801	1.58	5.88E-05	NIT (4)	2 (50%)
NIT8730	AGAP012379	1.65	6.85E-07		
Tubulin					
TubulinA	AGAP001219	6.98	3.00E-15	Tubulin (2)	2 (100%)
TubulinB	AGAP010510	2.99	6.03E-15		
GP					
GPRNPY3	AGAP012378	3.77	9.23E-16	GP (7)	2 (28%)
GPXH1	AGAP004247	2.10	3.08E-09		
Other					
AMYL	AGAP002317	5.35	1.49E-19	AMYL (1)	1 (100%)

CAT1	AGAP004904	5.31	5.91E-26	CAT (1)	1 (100%)
Actincytopl	TC48694 (TIGR)	5.27	1.25E-14	Actin (2)	1 (50%)
ABCC11	AGAP008436	5.07	3.92E-20	ABCC (5)	1 (20%)
XD18014	AGAP006220	4.04	4.15E-13	XD (3)	1 (33%)
AD20590	AGAP010499	3.64	5.72E-10	AD (2)	1 (50%)
NADPH_P450_red	AGAP000500	3.62	7.32E-11	NADPH_P450_red (1)	1 (100%)
TRXR	AGAP000565	2.80	3.78E-09	TRX (4)	1 (25%)
GSG8	AGAP010647	2.20	2.47E-09	GSG (1)	1 (100%)
AP7862	AGAP007809	2.04	6.67E-05	AP (1)	1 (100%)
RPS26	AGAP012100	1.81	3.45E-11	RPS (1)	1 (100%)
Xanthdehydro	4A3A-AAK-G-02-R (EMBL)	1.55	7.57E-07	Xanthdehydro (1)	1 (100%)
SOD3A	AGAP010347	1.50	8.22E-05	SOD (4)	1 (25%)

* These values represent the number of gene families/class within each group differentially expressed. These values are also represented as a percentage of the total number of that class present on the slide.

Table 4 List of genes differentially expressed in 3-day old adults in comparison to first instar larvae

Gene Name	Vectorbase Accession Number	Fold Change	Adj. *P*-value	Gene families/class numbers present on slide	Number of genes within families/class differentially expressed *
AGM1	AGAP012401	2.52	1.04E-08	AGM (1)	1 (100%)
COI	DQ465331 (GB)	2.15	2.97E-07	COI (1)	1 (100%)
CYP4G17	AGAP000877	2.39	4.51E-12	CYP4 (30)	1 (3%)
JV2		4.31	1.14E-09	JV (2)	1 (50%)
MnSOD1	AGAP010517	1.6	6.17E-05	SOD (4)	1 (25%)

* These values represent the number of gene families/class within each group differentially expressed. These values are also represented as a percentage of the total number of that class present on the slide.

Table 5 List of genes differentially expressed in fourth instar larvae in comparison to 3-day old adults

Gene Name	Vectorbase Accession Number	Fold Change	Adj. *P*-value	Gene families numbers/class present on slide	Number of genes within families/class differentially expressed *
Cytochrome P450 monooxygenase					
CYP325D1	AGAP002206	3.94	1.34E-17	CYP3 (30)	1 (3%)
CYP4C28	AGAP010414	2.19	6.98E-11	CYP4 (30)	3 (10%)
CYP4C36	AGAP009241	1.79	8.82E-18		
CYP4H24	AGAP000088	4.00	2.62E-17		
CYP6Y1	AGAP008208	1.75	2.51E-06	CYP6 (29)	2 (6%)
CYP6Y2	AGAP008207	1.54	2.32E-11		
CYP9J3	AGAP012291	2.54	1.02E-08	CYP9 (9)	3 (33%)
CYP9J5	AGAP012296	1.88	1.00E-18		
CYP9M1	AGAP009374	1.50	4.79E-05		
Esterase					
COEAE1A	AGAP001722	3.94	4.01E-11	COEAE (16)	5 (31%)
COEAE1F	AGAP006227	1.70	1.08E-07		
COEAE2A	AGAP001723	1.73	3.42E-05		

COEAE3G	AGAP006724	2.05	1.39E-10		
COEAE5O	AGAP002391	1.69	5.34E-07		
COEBE2C	AGAP005371	2.21	4.08E-06	COEBE (4)	1 (25%)
Glutathione S-transferase					
GSTD1-3	AGAP004164	1.81	6.56E-09	GSTD (15)	4 (26%)
GSTD10	AGAP004383	2.84	8.28E-11		
GSTD11	AGAP004378	1.54	4.38E-11		
GSTD3	AGAP004382	2.08	4.17E-15		
GSTMS3	AGAP009946	1.76	2.30E-09	GSTM (3)	1 (33%)
GSTS1-2	AF513639 (GB)	2.78	5.56E-24	GSTS (2)	1 (50%)
GSTU1	AGAP000947	1.97	3.04E-12	GSTU (3)	1 (33%)
Tubulin					
TubulinA	AGAP001219	1.73	2.75E-05	Tubulin (2)	2 (100%)
TubulinB	AGAP010510	3.78	2.05E-25		
GP					
GPXH1	AGAP004247	1.62	5.34E-07	GPX (7)	2 (28.6%)

GPRNPY3	AGAP012378	1.51	1.01E-05		
Other					
JV1		2.72	1.82E-15	JV (2)	1 (50%)
HPX15	AGAP010810	2.05	1.29E-06	HPX (19)	1 (5%)
GRX1	AGAP011107	1.89	2.51E-09	GRX (3)	1 (33%)
NIT8537	AGAP003515	1.81	6.91E-13	NIT (4)	1 (25%)
AMYL	AGAP002317	1.59	1.29E-07	AMYL (1)	1 (100%)
RPL19	AGAP004422	1.51	9.05E-06	RPL (1)	1 (100%)
ACE1	AGAP001356	1.77	1.52E-10	ACE (2)	1 (50%)
SOD2	AGAP005234	2.39	6.36E-11	SOD (4)	1 (25%)
SP11706	AGAP004014	2.40	5.61E-06	SP (9)	1 (11%)

* These values represent the number of gene families/class within each group differentially expressed. These values are also represented as a percentage of the total number of that class present on the slide.

Table 6 List of genes differentially expressed in 3-day old adults in comparison to fourth instar larvae

Gene Name	Vectorbase Accession Number	Fold Change	Adj. *P*-value	Gene families/class numbers present on slide	Number of genes within families/class differentially expressed *
Cytochrome P450 monooxygenase					
CYP6AK1	AGAP010961	1.69	1.08E-07	CYP6 (29)	1 (3%)
CYP6Z1	AGAP008219	1.87	1.94E-10		
Other					
AGM1	AGAP012401	2.85	9.09E-14	AGM (1)	1 (100%)
CPLC8	AGAP008446	2.25	4.56E-08	CPLC (1)	1 (100%)
Cytochrome_C	AGAP009537	2.04	1.74E-12	Cytochrome (2)	1 (50%)
JV2		5.24	1.36E-22	JV (2)	1 (50%)
TPX2	AGAP011054	2.06	1.03E-14	TPX (5)	1 (20%)

* These values represent the number of gene families/class within each group differentially expressed. These values are also represented as a percentage of the total number of that class present on the slide.

APPENDIX C

Published manuscript I

Christian, R. N., T. S. Matambo, B. L. Spillings, B. D. Brooke, M. Coetzee, and L. L. Koekemoer. 2011. Age-related pyrethroid resistance and P450 gene expression in the major African malaria vector, *Anopheles funestus* (Diptera: Culicidae). Accepted by Genet Mol Res.

Contribution:

I carried out the lab work, interpretation of all results and wrote the first and subsequent drafts of the manuscript.

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Age-related pyrethroid resistance and P450 gene expression in the major African malaria vector, *Anopheles funestus* (Diptera: Culicidae)

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Abstract

Anopheles funestus is a major vector of malaria in most of the African region. Resistance to pyrethroid and carbamate insecticides has been recorded in populations of this species in South Africa and Mozambique. The P450 gene, *CYP6P9*, has been shown to be highly transcribed in a permethrin (pyrethroid) resistant laboratory strain FUMOZ-R, originating from southern Mozambique. The aim of this study was to determine the relationship between pyrethroid resistance and gene transcription levels of two closely related genes, *CYP6P9* and *CYP6P13*, by adult mosquito age in FUMOZ-R. Levels of resistance to 0.75% permethrin were determined based on standard WHO insecticide susceptibility assays using females and males of different ages ranging from 3 to 30 days old. The transcription levels of the two genes were quantified using qPCR for each age cohort. The WHO insecticide susceptibility assays showed that percentage survival in males and females significantly decreased as age increased. Quantitative analysis of the two genes *CYP6P9* and *CYP6P13* showed the highest levels of expression at 10 days of age. There was no correlation between expression of these two genes and pyrethroid survival by age.

Key words: Anopheles funestus, age, pyrethroid resistance, P450 expression

Introduction

Malaria affects an estimated 247 million people worldwide resulting in approximately one million deaths each year (WHO 2008). Ninety percent of deaths due to malaria occur in Africa. Malaria is transmitted by *Anopheles* mosquitoes including the major African vector *Anopheles funestus,* which transmits malaria perennially and has also been associated with malaria epidemics (Gillies and De Meillon 1968, Fontenille et al. 1990, Hargreaves et al. 2000).

Effective malaria vector control is potentially hampered by the development of resistance to insecticides. Therefore, an understanding of resistance mechanisms in vector populations enables the development of resistance management strategies. The most common modes of insecticide resistance are reduced target-site sensitivity and enzyme mediated detoxification. Target-site insensitivity mutations result in reduced affinity of the target receptor to insecticide (Brogdon and McAllister 1998). Alterations of the sodium ion channel gene (*kdr*), the γ amino-butyric acid (GABA) receptor and acetylcholinesterase (AChE) reduce the binding affinities of pyrethroids and DDT, dieldrin and fipronil, and carbamates and organophosphates respectively (see review by Hemingway and Ranson, 2000).

The first account of insecticide resistance in *An. funestus* in southern Africa and its impact on malaria transmission was reported in 2000 (Hargreaves et al. 2000). Pyrethroid resistance in *An. funestus* from South Africa and southern Mozambique was subsequently showed to be mediated by a monooxygenase (P450) detoxification mechanism and no target site insensitivity was identified (Brooke et al. 2001, Wondji et al. 2007, Okoye, 2008). Recently, the molecular characterisation of monooxygenase based pyrethroid resistance in *An. funestus* was initiated (Amenya et al. 2005, Wondji et al. 2007, Amenya et al. 2008, Wondji et al. 2009, Matambo et al. 2010).

Amenya et al. (2005) isolated the first 31 partial monooxygenase P450 sequences and subsequently reported that one of these, *CYP6P9*, was over-expressed in the egg and adult stages of a pyrethroid resistant laboratory strain (FUMOZ-R) originating from southern Mozambique (Amenya et al. 2008). Wondji et al. (2007) identified a quantitative trait locus (QTL) associated with pyrethroid resistance in the same *An. funestus* strain and confirmed the increase in *CYP6P9* transcription. These QTL markers contained a cluster of P450 genes including *CYP6P9*. Subsequent work by Matambo et al. (2010) and Wondji et al. (2009) reported on a duplicate gene and called these genes *CYP6P13* and *CYP6P9b* respectively. Amino acid similarities between *CYP6P13* and *CYP6P9*b confirm that these are the same genes (Wondji et al. 2009, Matambo et al. 2010). As *CYP6P13* was officially named by the P450 Nomenclature committee *(*Dr Nelson, http://drnelson.utmem.edu/CytochromeP450.html) during 2009 (Matambo, 2008) this paper will retain the name *CYP6P13* for this gene.

Gene transcription studies by Amenya et al. (2008) and Wondji et al. (2009) did not differentiate between these two closely related genes as the primers used for both these studies were designed in a region sharing 100% sequence identity between them. Recent studies by Morgan et al. (2010) confirmed the increased mRNA expression of both these genes in *An. funestus* from Uganda while Cuamba et al. (2010) reported on the overexpression of one of the

duplicate genes (*CYP6P9a*, called *CYP6P13* in this article). Furthermore, a recent microarray study by Christian et al. (2010) showed an increase in gene transcription for these genes. Only one other P450 gene was found to be upregulated (*CYP6M7*) but this gene was not validated using quantitative real-time PCR (qPCR) (Christian et al. 2010).

As mosquitoes are infective and able to transmit malaria only after age 10-14 days, it is important to characterise insecticide resistance in older mosquitoes. The aim of this project was to quantify the levels of pyrethroid resistance in a highly resistant strain of *An. funestus* males and females at different ages (3, 5, 10, 14, 20 and 30 days) as well as to quantify the transcription levels of the two duplicated genes, *CYP6P9* and *CYP6P13,* in the same age cohorts in order to ascertain whether there is a direct relationship between gene transcription and pyrethroid resistance phenotype by age.

Materials and Methods

Mosquitoes:

The FUMOZ-R *An. funestus* laboratory colony originates from southern Mozambique and was selected for high levels of resistance to permethrin (pyrethroid). Details of colony rearing, permethrin resistance selection and insectary conditions can be found in Hunt et al. (2005). Female and male adults were separated on emergence and were maintained on a 10% sugar solution until they reached the ages of 3, 5, 10, 14, 20 or 30 days. A separate cohort of female adults were allowed to mate after emergence and were offered blood meals when they were six, eight and 10 days of age. On day 14 they were used either for insecticide susceptibility testing or for RNA extractions.

Evaluation of the influence of *An. funestus* age on their pyrethroid resistance:

The insecticide susceptibility assays described in this study were carried out according to WHO specifications using WHO insecticide treated papers (WHO 1998). The insecticidal action of all test papers was first confirmed using samples of an *An. funestus* susceptible strain (FANG), which showed 100% mortality following exposure to all papers.

Approximately 30 FUMOZ-R adults per replicate were assayed against 0.75 % permethrin treated papers through 10 replicates for each age and sex cohort (total of 300 mosquitoes per age group). For each replicate an untreated control was included. Final mortalities were recorded 24 h post exposure and subsequent analysis was conducted on these data (WHO, 1998). As sample sizes were large throughout (n=<290), data were not transformed prior to analysis.

Quantification of *CYP6P9* and *CYP6P13* transcripts for each age cohort:

Total RNA extraction and cDNA synthesis: Total RNA was extracted from 15 adult *An. funestus* FUMOZ-R mosquitoes to form one biological sample. Three biological extractions (n= 15 mosquitoes per biological sample) were performed per age and sex. The extractions were carried out using Tri reagent (Sigma, T9424, Munich, Germany). The RNA samples were DNase treated (Qiagen, 79254, West Sussex, UK), and were quantified using a Nanodrop ® Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Total RNA (up to 2µg) was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, 4387406, Johannesburg, SA). 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.

Primer Design:

Two sets of primers were used in this study, one for *CYP6P9* (gene-specific primers) and the other for *CYP6P13* (gene-specific primers). The ribosomal protein 7 or rps 7 (GenBank accession number: EF450776) primers were used as a reference gene against which to normalise data. This gene was tested prior analysis to confirm that transcription of this gene does not change with age.

The *CYP6P9* (GenBank accession number: EU450763) and *CYP6P13* (GenBank accession number: EF152577) primers were designed in the regions containing nucleotide base pair differences between the two genes and these primers are therefore specific for these genes. These primers were designed using the full-length *CYP6P9* and *CYP6P13* gene sequences (Matambo et al. 2010) (Table 1).

Real Time quantitative PCR (qPCR):

The qPCR reactions were carried out 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 standard curve reaction containing a 2-fold serial dilution of cDNA for each of the genes of interest and the house keeping gene was generated in each reaction. Serial dilutions of cDNA for the standard curve were prepared (Paton et al. 2000; Bio-Rad 2006). This was correlated to the initial cDNA template used in the reaction and then converted to transcript copy number (tCNR) (Bio-Rad 2006).

The cycling conditions for each primer set differed from each other. These conditions are presented in Table 1. Triplicates of each biological sample were carried out in each plate and the qPCR for the three different biological extractions was performed on different days. The reference gene, rps 7, did not vary between the different ages when amplified. The identity of all amplified products was confirmed by sequence analysis by sequencing in both directions. The sequenced genes were blasted against the *CYP6P9* and *CYP6P13* gene sequences to ensure that the correct product was amplified during qPCR.

The data were analysed using the absolute quantification method, using the formula, $N_n = 10$ (n-b)/ m, where n = Ct, b = y intercept, m = slope of line (Bio-Rad 2006). The values were converted into transcript copy numbers (tCNR) and the ratio of each gene to the rps 7 transcription was determined (Bio-Rad 2006). Standard errors (SE) were calculated for each replicate.

The Pearsons correlation statistical method was implemented to deduce if there was any association between the bioassay and the qPCR data.

Results

WHO Insecticide Bioassays:

Percentage survival of FUMOZ-R resistant adults 24 hours post exposure to 0.75% permethrin was calculated by gender for each age cohort (10 repeats of ±30 mosquitoes per tube) (Fig. 1). There was a significant trend in mortality with age in females (linear regression: $P= 0.04$; $R^2= 0.59$) and males (linear regression: $P= 0.02$; $R^2= 0.60$). In both cases survival tended to decrease with age. Three day old females showed a mean survival of 78.1% (SE: 8.48) which decreased steadily through ages 5 (71.71%; SE: 8.64), 10 (68%; SE: 7.96) and 14 days (37.70%; SE: 5.77), showing that female susceptibility to permethrin increased with age (Fig. 1A). The lowest survival was recorded in the blood-fed female cohort at 14 days (32.70%; SE: 7.26), although a two sample *t*-test revealed that there was no significant difference between blood-fed and unfed females at this age (P=0.64). Survival increased in the female cohorts at 20 and 30 days old. There was no significant difference between unfed 14 day and 20 day old females (P=0.08).

A similar trend was observed in the males (Fig. 1B). The males' resistance to permethrin was high at ages three (71.81%; SE: 3.97) and five days (77.86%; SE: 7.68). At 10 days the percentage survival decreased to 48.83% (SE: 8.85) and then steadily decreased to a low of 26.84% (SE: 5.13) at age 14. The lowest survival in the males was recorded at age 20 (17.40%; SE: 1.89). There was no significant difference between 14 day and 20 day old males (P=0.09).The percentage survival increased to 26.40% (SE: 4.28) at 30 days. There was a significant difference in percentage survival between three day and 14 day old males based on a two sample *t*-test (P=0.00).

Gene transcription analysis of *CYP6P9*:

In three day old females, the tCNR was 1.88 (SE: 0.40). In both pyrethroid resistant females and males, the tCNR was a high 9.3 (SE: 3.08) at day 10 for females and 20.1 (SE: 1.08) for males at the same age but decreased by day 14 to 3.63 (SE: 1.08) for females and 12.1 (SE: 3.46) for males (Fig. 2). However, the tCNR then decreased to 3.63 (SE: 1.08) for unfed

females and 1.74 (SE: 0.45) for blood-fed females at day 14, although there was no significant difference in tCNR between blood-fed and unfed cohorts (two sample *t*-test: $P= 0.18$). The tCNR increased again at day 20 to 3.54 (SE: 1.8) and then decreased to 0.52 (SE: 0.20) at day 30.

Male tCNR was 9.15 (SE: 3.87) at three days which increased to 23.9 (SE: 7.63) at five days followed by a steady decrease of 20.1 (SE: 1.08) at 10 days, 12.1 (SE: 3.46) at 14 days, 5.77 (SE: 1.83) at 20 days and 6.56 (SE: 2.02) at 30 days (Fig. 3). Overall, there was no significant difference in the absolute transcription level of *CYP6P9* in association with age (ANOVA: females, $P= 0.17$; males, $P= 0.07$). There was no significant correlation between survival (as quantified by insecticide bioassay) and gene transcription levels for *CYP6P9* (linear regression: females, $P= 0.46$, $R^2= 0.18$; males, $P= 0.16$, $R^2= 0.37$).

Gene transcription analysis of *CYP6P13:*

Gene transcription levels of *CYP6P13,* using the absolute quantification method, revealed that the tCNR changed only slightly from 4.69 (SE: 0.60) in three day old females to 4.82 (SE: 0.56) (Fig. 2) in 10 day old females, while males of the same age had a tCNR of 28.84 (SE: 4.88) (Fig. 3). However, the tCNR almost halved between three and 14 day old unfed females although the difference is not statistically significant (two sample *t*-test: $P= 0.15$). The tCNR of the gene had decreased from 2 (SE: 0.27) at 14 days when the females were not given a blood meal to a significant 0.38 (SE: 0.24) when the female had access to a blood meal ($P= 0.01$). The tCNR increased to 0.79 (SE: 0.32) by day 30 in females and had increased to 13.67 (SE: 0.53) in males from 8.92 (SE: 0.91) by age 20. Overall, there was no significant difference in the absolute transcription levels of *CYP6P13* in association with age (ANOVA: females, $P= 0.39$; males, $P= 0.6$). There was no significant correlation between survival and gene transcription levels for *CYP6P13* (linear regression: females, $P= 0.06$, $R^2= 0.62$; males, $P= 0.52$, $R^2= 0.11$).

Discussion

Variation in the expression of insecticide resistance with age is shown here for *An. funestus* and has also been described in other anophelines. Hunt et al. (2005) describe fluctuations in the expression of pyrethroid resistance with age in unselected and selected generations of FUMOZ-R. The effect of age may lead to a weakening of resistance mechanisms as a consequence of a general progressive decrease in physiological capacity (Hodjati and Curtis 1999). This could in turn result in the mosquitoes having reduced ability to respond or adapt to environmental changes.

Successive blood feeding prior to reaching 14 days old did not affect female susceptibility to pyrethroid intoxication as assessed four days after the last blood meal. However, Spillings et al. (2008) showed that blood-feeding significantly enhanced resistance to pyrethroid intoxication in the FUMOZ-R strain four hours post blood-feeding in three day old females using dose response curves. Hunt et al. (2005) showed that pyrethroid induced mortality was

significantly reduced in 14 day old and 20 day old blood-fed FUMOZ-R. These results collectively suggest that the expression of the resistance phenotype varies in response to fluctuations in physiological state, which is a function of various parameters including age, blood feeding history and mating status. The increase in survival in females at 20 days of age warrants further investigation.

Specific primers were designed for the individual genes *CYP6P9* and *CYP6P13*. The effect of age on gene transcription in the resistant strain FUMOZ-R was investigated in order to test for a correlation between insecticide survival and gene transcription levels. Bioassay data showed that permethrin induced mortality generally increased with age. In terms of gene transcription, the highest tCNR was observed in *CYP6P9* (9.30) and *CYP6P13* (4.82) at 10 days old. Permethrin survival of females was also highest between three and 10 days of age. However, statistical analysis revealed no significant overall correlation between gene transcription for either gene and susceptibility to permethrin. Further, the male tCNR's were higher than those of the females, in contrast to the bioassay data in which males showed a slightly lower survival than females. This discrepancy may be attributable to other biological functions (such as pheromone production) that P450s are associated with in males (Kasai and Tomita 2003).

Both CYP6P9 and CYP6P13 have shown increased mRNA expression in association with pyrethroid resistance (Amenya et al. 2008; Wondji et al. 2009, Morgan et al. 2010, Cuamba et al. 2010). However, the fold over expression for each varies depending on the origin of the test population. Further, it is recommended by WHO (WHO, 1998) that insecticide susceptibility studies be assessed using female mosquitoes aged 2-5days. As a consequence, this age cohort has also been used to identify the underlying insecticide resistance mechanisms. However, females as young as this cannot be malaria infective and are therefore not representative of that portion of a vector population responsible for malaria transmission. This is the first study to investigate variation in the frequency of resistance at different ages looking for correlations with gene transcription in a highly resistant strain of *An. funestus* from southern Africa. The results show clearly that there is no correlation with increase in phenotype mortality with age and P450 gene over-expression.

In conclusion, this study highlighted two important genes that have previously been shown to be associated with permethrin resistance in adults of southern African *An. funestus*. Both these genes showed transcription variation in association with mosquito age. However, transcript analysis provided only limited information as to how the function and effect of each enzyme varies with age. Future functional genomic studies will be necessary to clarify the role of these enzymes in pyrethroid metabolism. This is likely to prove complex as these enzymes may have multiple functions and their expression can be affected by numerous environmental as well as physiological factors. A recent analysis of field-collected *An. funestus* samples from Mozambique showed an association between pyrethroid resistance and the up-regulation of these two genes (Cuamba et al. 2010). However, this mode of resistance may not necessarily occur in other *An. funestus* populations.

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Figure legends

Fig. 1 Percentage survival by age of pyrethroid resistant *Anopheles funestus* (FUMOZ-R) in females (A) and males (B) 24 h post exposure to 0.75% permethrin. Standard errors (SE) are indicated on the bars. Control: Exposure of mosquitoes to untreated papers.

Fig. 2 *CYP6P9* and *CYP6P13* transcription analysis by age, expressed as copy number ratio, in pyrethroid resistant *An. funestus* FUMOZ-R females. Standard error bars are indicated.

Fig. 3 *CYP6P9* and *CYP6P13* transcription analysis by age, expressed as copy number ratio, in pyrethroid resistant *An. funestus* FUMOZ-R males. Standard error bars are indicated.

Table legends

Table 1 Primers used in Quantitative Real Time PCR

446 **Table 1 Primers used in Quantitative Real Time PCR**
447

Gene	Primer Sequence	Fragment Length (bp)	Annealing Temp (°C)	Melting Temp (°C)
CYP6P9 *(GAN: EU450763)	**Fwd** 5'AGA TGT GAT TGG CAC CTG T 3' **Rev** 5' TCG ATA TTC CAC CGT TTC CT 3'	232	55.00	82.00
CYP6P13 *(GAN: EF152577)	**Fwd** 5' CTG GAT CTC CTA ATT ATG ATG AAG TTT TTC 3' **Rev** 5' GTT CAC CGT CTC GCG GAC T 3'	132	59.10	81.00
rsp 7 *(GAN: EF450776)	**Fwd** 5' TTA CTG CTG TGT ACG ATG CC 3' **Rev** 5' GAT GGT GGT CTG CTG GTT C 3'	135	**	85.50

448
449 * GAN= Gene Accession Number
450 ** Annealing temperature for the rsp 7 gene was 55°C when testing for CYP6P9 and 59.10°C for CYP6P13
451 analysis.

APPENDIX D

Published manuscript II

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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 *A.* funestus are unknown, this study investigated the utility of the *Anopheles* gambiae 'detox chip' to screen for differentially expressed detoxification genes in *A. funestus*. Differential expression of detoxification genes in 3 day old adult females and males from the FUMOZ resistant strain and the FANG susceptible strain was identified using the *A.* 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 *A.* funestus strains.

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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 *Anopheles* funestus resistant to pyrethroids and carbamates in South Africa and Mozambique [7,8]. The development of pyrethroid resistance in A. 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 A. 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 Anopheles 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 A. gambiae populations from Kenya and Zanzibar [11], Benin [12], Nigeria [13] and Ghana [14,15] and has also been successfully used for A. arabiensis from Cameroon [16] and *Anopheles* stephensi from Dubai [17].

Biochemical assays and synergists have been used previously to characterize the resistance mechanisms in the FUMOZ-R

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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 A. gambiae 'detox chip' in determining potential genes associated with insecticide resistance in a southern African A. 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 A. 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 A. funestus adults were separated on day of emergence and fed on 10% sugar solution until they were 3 days old (without prior exposure to pyrethroids). The transcription levels in the 3 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 15 3 day old adult females or males using the PicoPure™ RNA Isolation kit (Arcturus) according to manufacturer's instructions. Amplified mRNA (amRNA) was synthesized using the RiboAmp™ 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 8 μg of amRNA for each of the samples 2 μl mRNA spike mix (Universal Lucidea Scorecard, Amersham), random hexamers (Invitrogen) and water was incubated for 5 min at 70 °C. 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 50 °C for 2½ h. A mixture of 1 M NaOH/20 mM 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 (<15 ng/μl) and poor dye incorporation (<0.1 pmol/μl of each dye) were discarded. A 5 μg 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 h at 30 °C and re-suspended in 15.5 μl of hybridization buffer (Corning). The targets were denatured by incubation at 95 °C for 5 min. The A. 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. [11]. The target mix was added to the microarray chip and hybridizations were performed at 38 °C for 16 h.

After the incubation the slides were washed using the Universal Hybridization kit (Corning). The slides were pre-soaked in wash solution 1 for 1–2 min at 38 °C, followed by a second wash of solution 1 for 2½ min and finally incubated in wash solution 2 at ambient temperature for 5 min. The slides were subjected to three washes in fresh wash solution 3 at ambient temperature for 1 min. 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 $\geqslant 1.5$ 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 3 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 No. EF152577) and *CYP6P9* (GenBank Accession No. 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 −70 °C 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 No. AY729660.1) and COI (Accession No. AY423059.1) genes using the A. funestus gene sequences. These primers were designed using Beacon Designer 3.0 software (Bio-rad, 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).

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 25 μl containing 12.5 μl IQ™ SYBR supermix (Bio-Rad, 1708882), 4 μl primer (2.5 μM), 1 μl cDNA (50 ng) 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.

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 3 day old adult females and males of the A. funestus FUMOZ-R (resistant) and FANG (susceptible) strains using the A. 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 42 to 38 °C and lower stringency wash buffers and shorter washing times were used. These optimized conditions were similar to those used for cross-species hybridization to A. 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 $\geqslant$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 A. gambiae 'detox chip', 1.18% of genes were significantly differentially expressed in the females as opposed to 9.45% in males. No genes were found to be over-expressed in both females and males from the susceptible A. funestus, FANG, strain. In the resistant A. funestus FUMOZ-R stain, however, three genes were differentially expressed in the females (Fig. 1). The gene with the highest fold change in expression in the females was a cytochrome P450 gene CYP6P9 (CYP6P3 orthologous gene in A. 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 A. gambiae) was 1.8-fold differentially expressed.

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) (Fig. 2). Other differentially expressed genes in the males are shown in Table 2.

Out of a total of 254 genes on the A. 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 A. funestus [18] enabling the degree of identity between the A. gambiae probes and their putative A. funestus orthologs to be determined. The probe sequences of the genes on the A. gambiae 'detox chip' were aligned against the known A. 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

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′	[a]	[a]	This paper
	Fwd2: 5′-TCA GAT TCC GAA AGA AAG C-3′ **Rev2:** 5′-ATC ACG ATG AAT CGC ATA C-3′	[a]	[a]	
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. [25]
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. [25]
rsp 7	**Fwd:** 5′-TTA CTG CTG TGT ACG ATG CC-3′ **Rev:** 5′-GAT GGT GGT CTG CTG GTT C-3′	135	[b]/85.50	Amenya et al. [31]

[a] No amplification.
[b] Annealing temperature for the rsp 7 gene is equivalent to the respective genes.

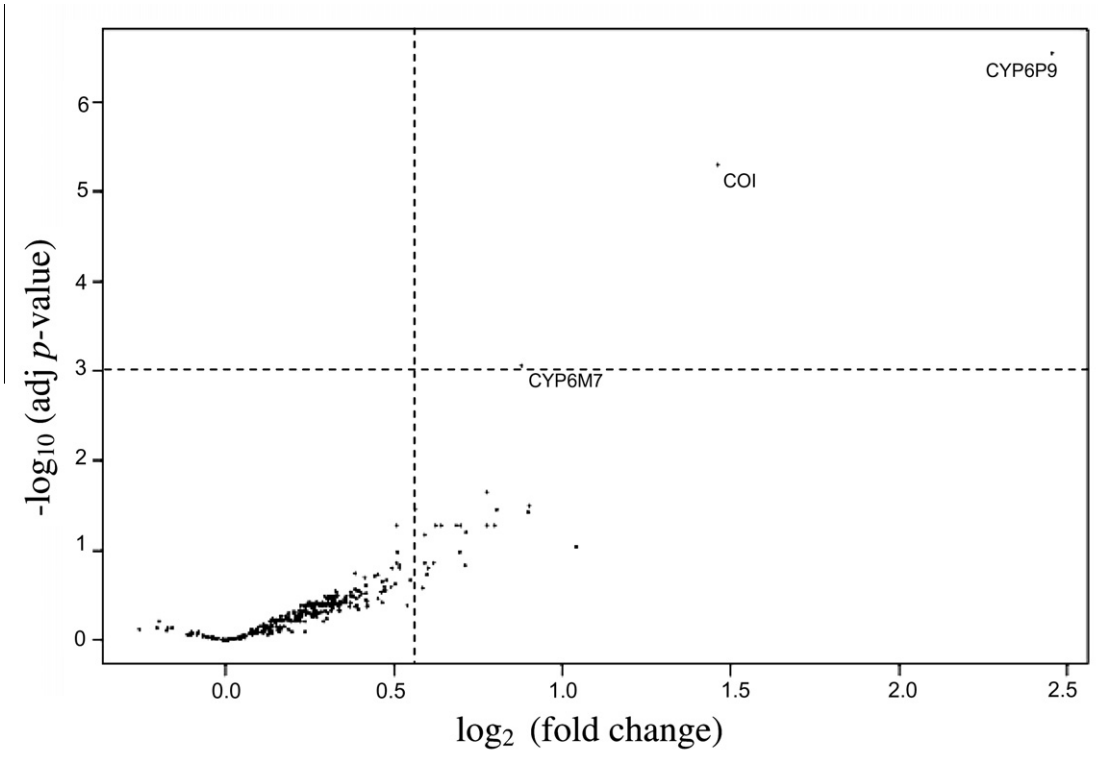

Fig. 1. Volcano plot representing genes differentially expressed in females of the *A. funestus* strain, FUMOZ-R. The horizontal line represents the cut off for the level of significance $\alpha = 0.001$ and vertical lines indicate cut off for the 1.5-fold change threshold.

Table 2
List of genes differentially expressed in females and males of the resistant *A. funestus* strain, FUMOZ-R. Gene names provided in table are as per *A. gambiae* naming, except for those where the *A. funestus* specific name has been identified (Amenya et al. [18]) which appears in brackets behind *A. gambiae* names.

Gene name	Group	Vectorbase Accession No.	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}
GSTS112	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}

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.

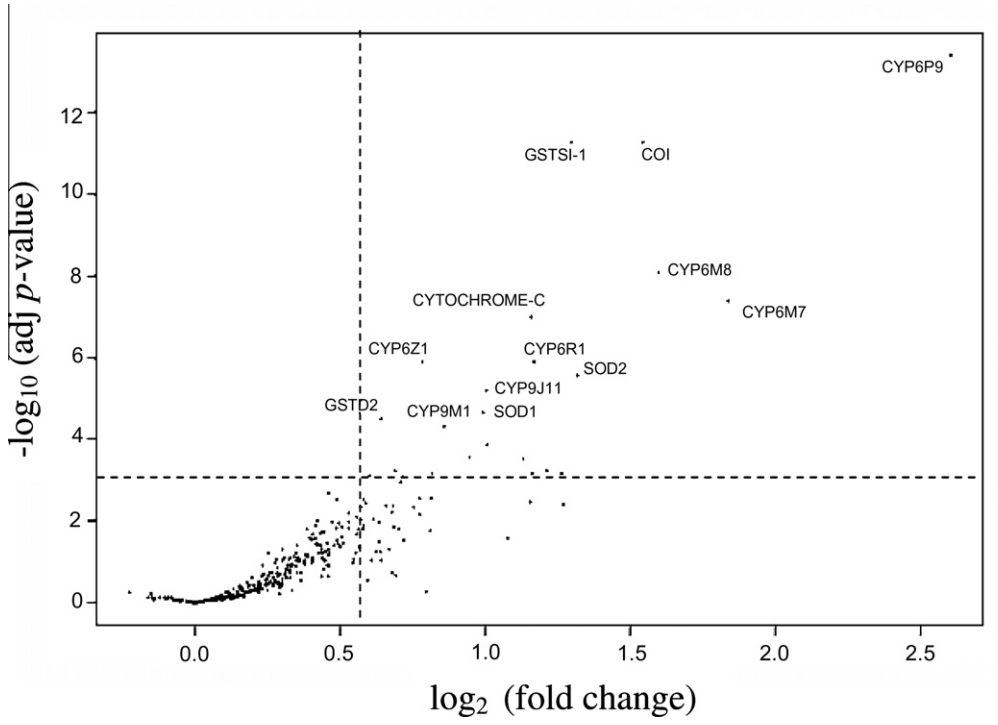

Fig. 2. Volcano plot representing genes differentially expressed in males of the *A. funestus* strain, FUMOZ-R. The horizontal line represents the cut off for the level of significance $\alpha = 0.001$ and vertical lines indicate cut off for the 1.5-fold change threshold.

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

Gene name	Group	Vectorbase Accession No.
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

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

A. funestus gene name	A. gambiae gene name	Percentage DNA similarity (%)	A. funestus gene Accession No.
COI	*COI*	50.3	AY423059.1
CYP6M1	*CYP6M1*	79.4	AY987356.1
CYP6M8	*CYP6M2*	49.4	AY729660.1
CYP6P4[a]	*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

[a] Indicates gene that did not produce any signal on the array.

The low level of sequence identity between some of the probes and their putative target genes in A. 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 by cross hybridization of probes to multiple genes cannot be excluded. Therefore qPCR was performed to validate the results from the genes found over-expressed in both sexes.

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 Fig. 3A and B, 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.

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 (Fig. 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 A. 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 A. funestus gene [18] (Table 1). However amplification of the product was unsuccessful for both primers even after numerous attempts.

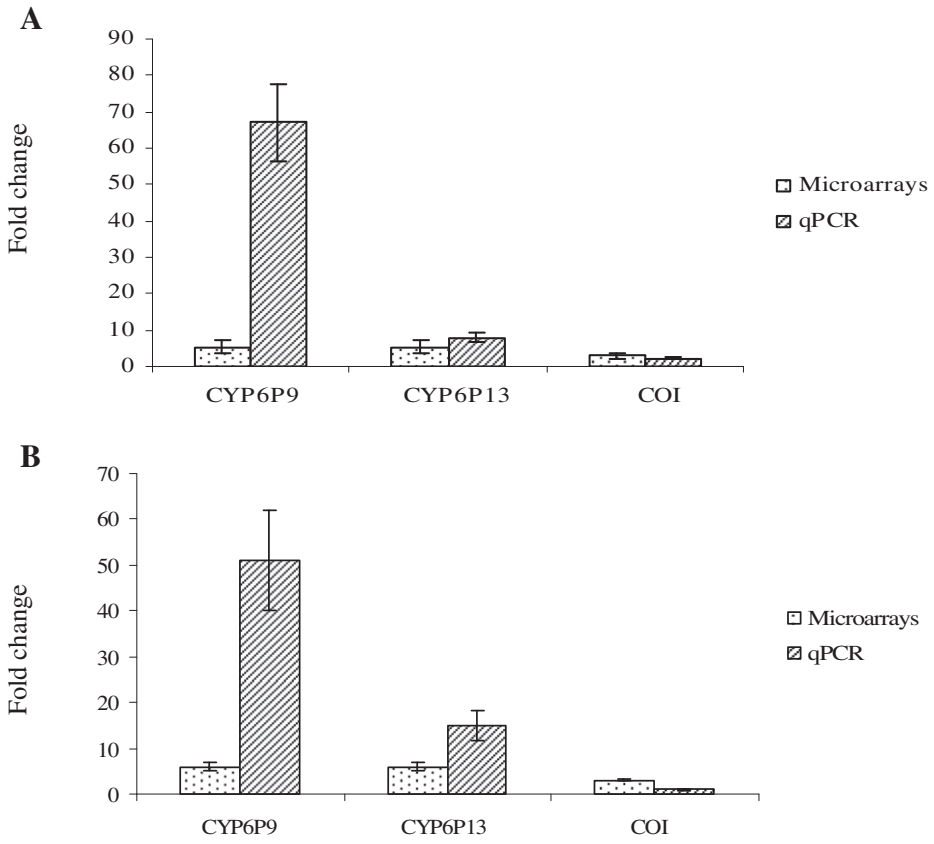

Fig. 3. Comparison of qPCR and microarray results for *CYP6P9*, *CYP6P13* and *COI* genes in females (A) and males (B).

4. Discussion

Gene expression in 3 day old A. funestus females and males was determined using the A. gambiae 'detox chip' developed by David et al. [11]. This study used cross-species (heterologous) hybridization of the A. funestus samples to an A. gambiae microarray platform due to the fact that unlike A. gambiae, a fully sequenced genome for A. 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 non-human 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 A. gambiae 'detox chip' has been successfully used to determine the transcriptional analysis of pyrethroid resistance in A. stephensi [17] and A. arabiensis [16]. This is the first study to employ microarrays to determine the genetic basis of pyrethroid resistance in A. 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 A. 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 A. 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 A. 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 A. 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 (A. gambiae ortholog) was also found to be differentially expressed (2.82-fold) in field caught A. gambiae adults from Ghana [15]. The CYP6P3 gene was also found at significantly high levels (2.6-fold) in A. 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) [33–35], thioredoxins (TRX) [36], glutathione-S-transferases (GSTs) [37–39].

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 A. gambiae and A. 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 A. 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 A. gambiae 'detox chip' provided valuable information in identifying potential genes involved in insecticide resistant in A. 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 A. 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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