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ROLE OF MONOOXYGENASES IN INSECTICIDE RESISTANT *ANOPHELES FUNESTUS* (DIPTERA: CULICIDAE)

by

Dolphine Achieng' Amenya

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2005

DECLARATION

I DOLPHINE ACHIENG' AMENYA declare that this thesis is my own, unaided 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 in any other University.



Signature of candidate

17th day of December, 2005

DEDICATION

To my loving mum and late dad. For their encouragement and support during my study period.

ABSTRACT

The widespread use of pyrethroid insecticides has led to the emergence of significant insecticide resistance in various parts of the world. An unprecedented increase in the number of annual malaria cases reported in Kwazulu Natal, South Africa in 1999 to 2000 was attributed to the re-emergence of pyrethroid-resistant *Anopheles funestus* Giles. Resistance was metabolic-based with increased monooxygenase (P450) metabolising the pyrethroid insecticides. This emphased the need to understand the molecular mechanisms conferring pyrethroid resistance in *An. funestus*. The present study aimed to firstly isolate P450 genes in *An. funestus* and secondly, to identify P450 gene over-expressed in a resistant (FUMOZ-R) strain compared to a susceptible (FANG) strain. A third aim was to construct an *An. funestus* cDNA library to lay the foundation for future studies on P450 monooxygenses.

Degenerate primers based on conserved regions of three *An. gambiae* P450 families were used to amplify cDNAs from *An. funestus*. Eleven CYP4, four CYP6 and five CYP9 partial genes were isolated and sequenced. BLAST results revealed that *An. funestus* P450s have a high sequence similarity to *An. gambiae* with above 75% identity at the amino acid level. The exception was CYP9J14. The *An. gambiae* P450 with the closest similarity to CYP9J14 exhibited only 55% identity suggesting a recent duplication event in CYP9J14. Molecular phylogenetic analysis also supported this hypothesis. Intron positions were highly conserved between the two species.

Expression studies using blot analysis implicated CYP6P9, an ortholog of CYP6P3 in *An. gambiae*, as the over-expressed P450. Dot blot analysis revealed a 500-fold expression higher in FUMOZ-R strain compared with FANG strain. Semi-quantitative PCR revealed that CYP6P9 was developmentally regulated. Expression was not detected in eggs and was higher in larvae compared to pupae. Quantitative real time PCR showed that CYP6P9 expression was 4.5-fold higher in 3-day old FUMOZ-R males than females and 3.5-fold higher in the 14-day old males than 14-day old females. Statistically, this difference was not significant suggesting that CYP6P9 expression is not sex specific.

The *An. funestus* cDNA library construction in λTriplEx2 vector was successful with a titre of 4.9 x10^8 pfu/ml and a transformation efficiency of 98%.

PUBLICATIONS

Amenya, D.A., Koekemoer, L.L., Vaughan, A., Morgan, J. C., Brooke, B. D., Hunt, R. H., Ranson, H., Hemingway J. and Coetzee M. (2005). Isolation and sequencing of P450s from a pyrethroid resistant colony of a major malaria vector *Anopheles funestus*. *DNA Sequence DNA Sequence* 16: 437-445. (See Appendix D).

Amenya, D.A., Koekemoer, L.L., Brooke, B. D., Hunt, R. H., Ranson, H., Hemingway J. and Coetzee M. Overexpression of CYP6P9 in a pyrethroid resistant *Anopheles funestus*. (*Manuscript in preparation*).

PRESENTATIONS

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African malaria vector An. funestus. South African Society of Biochemistry and Molecular Biology conference 16th-20th January, Stellenbosch University, South Africa. pp 86.

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ABBREVIATIONS

%	percent
µg	microgram
µl	microliter
µM	micro molar
$AgNO_3$	silver nitrate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
DDT	1,1,1,-trichloro-2,2,-bis (p-chlorophenyl) ethane/Diethyldiphenyltrichloroethane
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylene diamine tetra acid (disodium salt)
et al.	and others
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
g	relative centrifugal force
g/l	grams per liter
h	hour (s)
HCl	hydrochloric acid

ITS2	internal transcribed spacer 2
kb	kilo base
KCl	potassium chloride
KCl	potassium chloride
KOAc	potassium acetate
KOH	potassium hydroxide
M	molar
mg	milligram
$Mg(Oac)_2$	magnesium acetate
Mg_2SO_4	magnesium sulphate
$MgCl_2$	magnesium chloride
min	minute (s)
ml	milliliter
mm	millimeter
mM	millimole
MOPS	3-[N-morphilino] propane sulphonic acids
mRNA	messenger RNA
Na_2CO_3	sodium carbonate
NaAc	sodium acetate
NaCl	sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	the reduced form of NADP
NaOH	sodium hydroxide
ng	nanogram

nm	nanomole
NZY	NZ amine with yeast
^{o}C	degrees celsius
OD	optical density
P	probability level
PCR	Polymerase Chain Reaction
PLA2	phospholipase A2
pH	potential of hydrogen
pmol	picomole
$RbCl_2$	rubidium chloride
rDNA	ribosomal DNA
RNA	ribonucleic acid
rpm	revolutions per minute
s	second (s)
SMART	switching mechanism at 5' end of RNA transcript
SM1	salivary gland and midgut binding peptide 1
UTR(s)	untranslated region(s)
UV	ultra violet
v/v	volume per volume
vol	volume (s)
w/v	weight per volume
χ^2	chi-square

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Malaria is one of the leading impediments to human health in tropical Africa. There are 350-500 million acute cases of malaria worldwide each year with the majority occurring in sub-Saharan Africa (WHO, 2005). Approximately 1.5 to 2.7 million deaths occur annually, the bulk of which occur in sub-Saharan Africa where an estimated 360 million people live in areas of stable, endemic parasite transmission (Snow *et al*., 1999). Human malaria is an infectious disease caused by four species of the *Plasmodium* parasite namely, *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* and is spread by certain mosquitoes of the genus *Anopheles*. Of the four recognized human parasites, *P. falciparum* is the only species associated with severe morbidity and mortality. The other three species cause milder illness, however, infections with *P. ovale* and *P. vivax* may relapse months later if appropriate treatment is not provided (Bruce-Chwatt, 1963).

The principal vectors of malaria in Africa are *Anopheles gambiae s.s*. Giles, *An. arabiensis* Patton and *An. funestus* Giles (Gillies and De Meillon 1968; Gillies and Coetzee, 1987). Malaria transmission is intense in tropical Africa and major vector species may differ among geographical regions or change seasonally, making it difficult to determine which population is actually transmitting the

infective pathogen (De Meillon, 1947; Fontenille *et al.*, 1997; Minakawa *et al*., 2002).

Although the medical community has known for over a century the role mosquitoes play in the transmission of malaria, Africa continues to bear an overwhelming proportion of the malaria burden (WHO, 2005). The frequent epidemics and re-surgence of the disease in this region may be attributed to several factors. These include rapid spread of resistance of malaria parasites to anti-malarial drugs (Sweeney, 1996; Nchinda, 1998; WHO, 2004), wars, migration of non-immune populations from areas of low malaria transmission to areas of high malaria transmission, vector abundance and transmission potential caused by climatological changes (Mouchet, 1998) and insecticide resistance (Hargreaves *et al*., 2000). The development of both physiological and behavioural traits in vector populations which undermine vector control efforts particularly insecticides usage are additional factors (Collins and Paskewitz, 1995; Nchinda, 1998).

1.2 Malaria vectors

In sub-Saharan Africa, the three main vectors involved in transmission of malaria belong to two groups, namely, the *Anopheles gambiae* complex and the *Anopheles funestus* group. Below is a discussion of each group.

1.2.1 *Anopheles gambiae* complex

The *An. gambiae* complex consists of seven recognised species that are morphologically similar but differ in behaviour, feeding preferences and breeding requirements (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Hunt *et al*., 1998). Species within this complex include the major vectors *An. gambiae s.s*. and *An. arabiensis*, the minor vectors *An. merus* Dőnitz, *An. melas* Theobald and *An. bwambae* White, and the non-vectors *An. quadriannulatus* (Theobald) species A and B (White, 1972; Zahar 1985; Hunt *et al*., 1998). *Anopheles merus* was once considered an inefficient vector (Mosha and Petrarca, 1983). However, it has been shown to be as important as other members of the complex in malaria transmission but in localized areas (Temu *et al*., 1998; Masendu *et al.,* 2005).

1.2.2 *Anopheles funestus* group

The mosquitoes are small in size, dark and show uniformity in the adult morphology (Gillies and De Meillon 1968; Gillies and Coetzee, 1987). The members of this group comprise of ten species, nine of which are found in the Afrotropical region and the tenth species, *An. fluviatilis* James, recorded from the Arabian peninsula (Gillies and De Meillon 1968; Gillies and Coetzee, 1987). Four of the species *An. funestus*, *An. aruni* Sobti, *An. parensis* Gillies and *An. vaneedeni* Gillies and Coetzee, have almost identical morphology at all life stages and are referred to as the Funestus subgroup (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Constantini *et al.*, 1999; reviewed in Coetzee and

Fontenille, 2004). Other than these four, other species within the group include *An. leesoni* Evans, distinct at both the egg and larval stages, *An. confusus* Evans and Leeson easily identified on larval characteristics, *An. rivulorum* Leeson and *An. brucei* Service with distinctive larvae although the two species are indistinguishable from each other (Gillies and De Meillon, 1968). The ninth species, *An. fuscivenosus* Leeson, is known only from the adult stage (Gillies and De Meillon, 1968; reviewed in Coetzee and Fontenille, 2004).

1.2.2.1 *Anopheles brucei* Service

The type locality for *Anopheles brucei* is Lokoja, northern Nigeria. The species closely resembles *An. rivulorum* both in its larval and pupal stages but can be differentiated in the adult stage based on features of the palps, pharynx, mesonotum and wings (Gillies and De Meillon, 1968). So far, there is little information on the biology of this species.

1.2.2.2 *Anopheles confusus* Evans and Leeson

Anopheles confuses is localised, in southern Africa being widespread in Zimbabwe and the north-eastern South Africa lowveld (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The larvae are found in slow moving waters but White (1972) reported larvae from hippopotamus footprints. This species is presumed to be zoophilic.

1.2.2.3 *Anopheles fuscivenosus* Leeson

Anopheles fuscivenosus is described from Shamva in Rhodesia (Zimbabwe). The species resembles *An. funestus* but can be separated from the group by the dark wings. The early life stages are unknown and the only information recorded is the fact that collections were from outdoor resting-sites (Gillies and De Meillon, 1968).

1.2.2.4 *Anopheles leesoni* Evans

Anopheles leesoni was described from Rhodesia (Zimbabwe) with no precise location mentioned. The early stages are quite different from other members of the group (Gillies and De Meillon 1968). The larval habitat is similar to *An. funestus* except that it is found in the edges of slow flowing streams. Adults are found in houses but collections are common in natural resting sites and the species is presumed to be zoophilic with no role in malaria transmission (Gillies and De Meillon, 1968). Molecular studies have revealed a close phylogenetic relationship between *An. leesoni* and the Asian *An. minimus* group at the ITS2 region with a one base difference from the ITS sequence of *An. leesoni* (Garros *et al.* 2004).

1.2.2.5 *Anopheles rivulorum* Leeson

Anopheles rivulorum has its type locality recorded as Sinoia, Rhodesia (Zimbabwe). The species is mainly distributed in the eastern and southern Africa

from Ethiopia through Zimbabwe, Mozambique, Botswana and South Africa. Localized situations have been reported in West Africa from Mali, Côte d'Ivoire to northern Nigeria (Gillies and De Meillon, 1968). Most of the life stages in this species differ from the rest of the group. It is both exophilic and zoophilic but occasionally found in houses (Gillies and De Meillon, 1968). Although Gillies and Smith (1960) recorded no infections in the salivary glands of female *An. rivulorum*, in Tanzania, Wilkes *et al*., (1996) showed that it was playing a role in *Plasmodium falciparum* transmission. The species is currently regarded as a minor malaria vector.

1.2.2.6 The Funestus subgroup

Anopheles funestus within this subgroup have almost identical morphology at all life stages (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Constantini *et al.*, 1999; reviewed in Coetzee and Fontenille, 2004).

1.2.2.6a *Anopheles aruni* Sobti

Anopheles aruni was recorded from Kaburi Kikombero on Zanzibar island. The species is indistinguishable from *An. funestus* both in the larval and pupal stages. However, the broad palpal bands and pale wings found in the females have been used to distinguish it from *An. funestus*. The only information available on the adult behaviour is the fact that female *An. aruni* attack humans outside at night

and both males and females can be caught resting by day in shaded banks and tree bases (Gillies and De Meillon, 1968).

1.2.2.6b *Anopheles vaneedeni* Gillies and Coetzee

Anopheles vaneedeni was first described from Pusela, Tzaneen in South Africa. Larval habitats are quite similar to that of *An. funestus* and the species shows little difference from *An. funestus* making morphological separation difficult**.** *Anopheles vaneedeni* mainly feeds on animals but will feed on humans outdoors especially in the early hours of the night. Although, laboratory evidence showed that it could transmit *P. falciparum*, its involvement in malaria transmission remains uncertain (De Meillon *et. al*., 1977; Gillies and Coetzee, 1987).

1.2.2.6c *Anopheles parensis* Gillies

Anopheles parensis was recorded from Kihurio, South Pare district in Tanzania. It is mainly distributed in the lowlands from the Kenyan coast, north-eastern Tanzania, Pemba Island, Swaziland and South Africa. The egg, larval and adult stages resemble those of *An. funestus*. The larvae are found in permanent swamps and ponds among reeds and emergent vegetation (Gillies and De Meillon, 1968). *Anopheles parensis* was first recognized in Tanzania after residual house-spraying led to the elimination of *An. funestus*. This species is not capable of malaria transmission in the presence of insecticides and in the absence of *An. funestus*, it is of little or no importance (Gillies and De Meillon, 1968). Recently in Mwea

Kenya, *An. parensis* was the most common member of the *An. funestus* group found resting in human dwellings but was not implicated in malaria transmission (Kamau *et al.,* 2003). *Anopheles parensis* was also found resting indoors in Mamfene, South Africa in large numbers but none was found to be infected (L. Koekemoer, personal communication).

1.2.2.6d *Anopheles funestus* Giles

Anopheles funestus is one of the most important vectors of malaria in sub-Saharan Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The larvae of this species develop in permanent or semi-permanent pools and are less dependent on rainfall unlike the *An. gambiae* complex (Gillies and De Meillon 1968). The biology of the species has been extensively studied compared to other members in the group. *Anopheles funestus* has been found to be one of the most anthropophilic mosquitoes, feeding on humans even in the presence of alternative hosts such as domestic animals. *Anopheles funestus* was considered to be eurygamic and therefore refractory to colonization (Gillies and De Meillon, 1968) but this has been shown to be incorrect with two colonies currently housed in the National Institute for communicable Diseases (NICD), Johannesburg (Hunt *et al*., 2005).

Biting activity of this species takes place in the second half of the night with the great bulk of feeding indoors (Gillies and De Meillon, 1968). Adults spend most of their life in houses making it vulnerable to residual insecticides. In the absence

of control measures, female mosquitoes have been found infected with sporozoites wherever adequate numbers exist (Gillies and De Meillon, 1968). Other than its role in malaria transmission, *An. funestus* has also been shown to transmit other diseases such as Bancroftian filariasis and Tanga virus (Gillies and De Meillon, 1968; Awolola *et al*., 2005).

1.2.2.7 Species identification

Proper species identification is an essential step to separate vectors from non-vectors which is important in the implementation of vector control strategies (Koekeomer *et al*., 2002).

Polytene chromosomes found in the salivary glands of fourth instar larvae and in nurse cells of half gravid ovaries of females have been useful in mosquito identification (Green, 1982). Green and Hunt (1980) characterised three members of the *An, funestus* group from southern and east Africa using polytene chromosomes. *Anopheles vaneedeni* was found to have homosequential banding arrangements with *An. funestus*, differing by a single floating inversion on arm 2. Chromosome of *An. parensis* was shown to differ from that of *An. funestus* and *An. vaneedeni* by one fixed inversion on arm 3 (Green and Hunt, 1980; reviewed in Coetzee and Fontenille, 2004). Chromosomal maps for other members of the *An. funestus* group, *An. rivulorum*, *An. confusus*, *An. fuscivenosus* and *An. leesoni* revealed that *An. leesoni* was genetically distinct and was more closely related to

the oriental *An. minimus* group (Green, 1982; reviewed in Coetzee and Fontenille, 2004).

The development of molecular tools as a means of species identification is a relatively rapid, inexpensive technique, applicable to all life stages and sex with limited starting material required. One of the first molecular-DNA based method used to distinguish between *An. funestus* and *An. vaneedeni*, was restriction fragment length polymorphism (RFLP) (Koekemoer *et al*., 1998). Polymerase chain reaction – single strand conformation polymorphism (PCR-SSCP) (Koekemoer *et al*., 1999) followed the RFLP assay. This method clearly identified *An. funestus*, *An. leesoni* and *An. rivulorum* but could not distinguish between *An. vaneedeni* and *An. parensis*. Currently, a rapid rDNA (PCR) assay based on the species-specific primers in the ITS2 region of *An. funestus* is used to accurately identify five members of this group namely, *An. funestus*, *An. vaneedeni*, *An. rivulorum*, *An. leesoni* and *An. parensis* (Koekemoer *et al*., 2002). Garros *et al.*, (2004) developed a multiplex PCR assay with additional primers to identify members of the *An. funestus* and the oriental *An. minimus* groups.

1.3 Vector control

Vector control remains one of the most generally effective measures to prevent malaria transmission and as such, it is one of the four basic technical elements of the global malaria control (WHO, 2005). Vector control programmes have focused on indoor house spraying using residual insecticides and the use of

insecticide treated bed nets for personal protection. In Sri Lanka, for example, synthetic insecticides have been the major tools in mosquito control programmes for decades after the phasing out of the use of DDT in the late 1970s (Karunaratne and Hemingway, 2001). However, reports have since shown malathion resistance in *An. culicifacies*, *An. subpictus* and *Culex tritaeniorhynchus* (Karunaratne and Hemingway, 2001). Biological control using larvivorous fish and environmental management of mosquito breeding sites has also been employed with limited success (Garrett and White, 1977, Coluzzi, 1992; WHO, 2005). The achievements of these methods are recognized with varying degrees (Coluzzi, 1992). Presently, WHO has allowed for the restricted and controlled use of DDT in disease vector control, for example in situations where insecticide resistance is a problem (Hargreaves *et al*., 2000).

Vector control research has also focused on the concept of permanently altering vector competence by creating a parasite-refractory mosquito from a susceptible population (Curtis, 1968; Collins *et al*., 1986; Beerntsen *et al.,* 2000). This began when a susceptible strain of *Aedes aegypti* was rendered refractory to dengue virus using the Sindbis virus to express an antisense RNA complementary to part of the dengue virus genome (Olson *et al*., 1996). However, this Sindbis virus-mediated expression system was found to be transient and not heritable.

Efforts to transform mosquitoes have focussed on the use of transposable elements such as *Hermes* and *piggyBac*, which were isolated initially from *Musca domestica* and *Trichoplusia ni* respectively (O'Bronchta *et al.*, 1996; Fraser *et al*.,

1996). It is worth noting that although a number of candidate molecules can serve as anti-pathogen gene products, each molecule shows some advantages and disadvantages. This must be critically assessed to find an antipathogen molecule whose production creates the least possible physiological stress on the mosquito (Beerntsen *et al*., 2000). The genetic background of mosquitoes in terms of fitness has also been shown to play an important role in experiments gearing towards genetic manipulations (Moreira *et al*., 2004). For example, mosquitoes transformed with SM1 construct, a tetramer of the SM1 dodecapeptide gene from honeybee venom, had no significant reduction in fitness parameters in comparison to those that had the PLA2 construct (Moreira *et al*., 2004).

1.4 Insecticide Resistance

Resistance has been defined as "the inherited ability of a strain of some organisms to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species" (adapted from Scott, 1999). Resistance to insecticides hinders the control of agricultural and medically important arthropods and has been reported in various parts of Africa since the 1950s (Davidson, 1956; Brown, 1986; Coetzee *et al.,* 1999; Kasai and Scott, 2000; Sina and Aultman, 2001). Although the mechanisms by which insecticides become less effective are similar across all vector taxa, each resistance problem is potentially unique and may involve a complex pattern of resistance foci (Brogdon and McAllister, 1998). It is therefore necessary to outline factors which account for the toxic action of the particular insecticide. Insecticide toxicity depends on factors such as (a)

penetration of the chemical through the insect's integument, (b) penetration at the site of action, (c) enzymatic conversion of the chemical to more toxic compounds, (d) enzymatic conversion to less toxic metabolites, (e) excretion of the insecticide and or toxic metabolites and (f) storage of the insecticide or toxic metabolites in non-sensitive tissues (Perry, 1966; Zlotkin, 1999).

Increased tolerance to an insecticide may be due to genetic selection or enhancement of those processes favouring survival of the individual organism (Perry, 1966). Studies have uncovered the gene-protein relationships that are important in investigating the physiology of gene action in insects such as mosquitoes (Klassen, 1966, Hemingway *et al*., 1998, Martinez-Torres *et al*., 1998).

Today, the major emphasis on research into resistance focuses on molecular mechanisms and rational resistance management with a view to controlling the spread and development of resistance in vector populations (reviewed in Hemingway, 2003). The level of resistance in insect populations is dependent on the amount and the frequency of insecticides used as well as the inherent characteristics of the insect species selected. For instance, mosquitoes have all the characteristics suited for rapid development of resistance including short life cycles and abundant progeny (reviewed in Hemingway, 2003).

The mechanisms of resistance found in insects are discussed in the next section.

1.4.1 Target site insensitivity

Decreased sensitivity of all major insecticide target sites, such as acetylcholinesterases (AChEs), the voltage-sensitive sodium channel and gama-aminobutyric acid (GABA)-gated chloride channel, have been reported in insects (McCaffery, 1998, Vais *et al*., 2001). To date, reported modifications have been exclusively point mutations of structural genes encoding the target site protein (ffrench-Constant, 2000). Target-site insensitivity to an insecticide usually confers cross-resistance to other compounds that share the same target site. For example, decreased sensitivity of the sodium channel to diethyldiphenyltrichloroethane (DDT) confers cross-resistance to pyrethroids, which share the same target site (Brogdon *et al*., 1999; Chandre *et al*., 1999).

1.4.1.1 Sodium ion channels (Knock down resistance-*kdr*)

1,1,1,-trichloro-2,2,-bis (p-chlorophenyl) ethane (Diethyldiphenyltrichloroethane, DDT) and pyrethroids cause persistent activation of the sodium ion channels by delaying the normal voltage-dependent mechanisms of inactivation (Soderlund and Bloomquist, 1989). In West and East Africa, there have been reports of suspected *kdr*-like resistance inferred from cross-resistance between DDT and pyrethroids in *An. gambiae* (Martinez-Torres *et al*., 1998; Chandre *et al*., 1999; Ranson *et al*., 2000a).

Kdr occurs due to a change in the affinity between the insecticides and its binding site on the sodium channel caused by a single or multiple substitutions in the sodium channel (Martinez-Torres *et al*., 1998; Ranson *et al*., 2000a; reviewed in Hemingway *et al*., 2004). In the pyrethroid resistant West African *An. gambiae*, the mutation is the leucine to phenylalanine (Leu→Phe) substitution in the S6 hydrophobic segment of domain II in the sodium channel gene (Martinez-Torres *et al*., 1998). However, in the East African *An. gambiae*, a second substitution at the same position involving leucine to serine (Leu→Ser) was found (Ranson *et al*., 2000a).

1.4.1.2 Gamma aminobutyric acid (GABA) receptors

Gamma aminobutyric acid (GABA) receptors are a widespread inhibitory neurotransmission channel in the central nervous system and in neuromuscular junctions. The insect GABA receptor is implicated as a site of action for ivermectins as well as cyclodienes (reviewed in Hemingway and Ranson, 2000). The GABA receptor-ionophore complex has also been implicated as a target site for Type II pyrethroids although the concentrations required to cause this action is much higher than that capable of disrupting the sodium ion channel (Soderlund and Bloomquist, 1989).

1.4.1.3 Acetylcholinesterase

The carbamates and organophosphates target acetylcholinesterase (AchE), resulting in the hydrolysis of neurotransmitter acetylcholine (Ach) to terminate neuronal excitement at the postsynaptic membrane (Nabeshima *et al*., 2004). The insecticides affect the enzyme by covalent phosphorylation and cabamylation of the serine residue within the active site gorge of the enzyme (reviewed in Hemingway *et al*., 2004). Alterations in AchE gene in resistant insects result in decreased sensitivity to insecticide (reviewed in Hemingway *et al*., 2004). The insensitive AchE has been reported in a number of *Anopheles* species such as *An. atroparvus* (Hemingway, 1982), *An. stephensi* (Hemingway, 1983), *An. albimanus* (Hemingway and Georghiou, 1983) and recently in *An. gambiae* (N'Guessan *et al.,* 2003).

1.4.2 Metabolic resistance

The enzymes responsible for detoxification of xenobiotics in living organisms are transcribed by members of large multigene families of esterases, oxidases and glutathione S-transferase (GSTs) (Terriere, 1984). In insects, metabolic insecticide resistance is associated with increased levels of cytochrome P450s (monooxygenases), GSTs, esterases or a combination of these genes (Hemingway and Karunaratne, 1998; Huang *et al*., 1998; Daborn *et al*., 2002; Nikou *et al*., 2003; Festucci-Buselli *et al*., 2005). Esterases are mainly involved in organophosphate, carbamate and to a limited extent in pyrethroid resistance

(reviewed in Hemingway and Ranson, 2000). Monooxygenases are mainly involved in the metabolism of pyrethroids and to a lesser extent, detoxification of organophosphates and carbamates (reviewed in Feyereisen, 1999). P-glycoproteins which are implicated in multiple drug resistance (MDR) functions as an ATP dependent extrusion pump for drugs and physiological substrates (Buss *et al*., 2002). GSTs on the other hand play a role in detoxification of a large range of xenobiotics such as to the organochlorine insecticide DDT. They also provide protection against oxidative stress and hormone biosynthesis (reviewed in Enayati *et al*., 2005).

1.4.2.1 Glutathione S-transferase (GSTs) based resistance

Glutathione S-transferases (GSTs) are a major family of detoxification enzymes, which possess a wide range of substrate specificities (reviewed in Enayati *et al*., 2005). Most organisms possess multiple GSTs belonging to two or more classes with differing catalytic activities to accommodate the wide range of substrate specificities (Chen *et al*., 2003). Insect GSTs are classified into six classes: Delta (δ), Epsilon (ε), Sigma (σ), Theta (θ), Omega (ω) and Zeta (ζ) with the δ and ε-classes implicated in detoxification of insecticides by dehydrochlorination (Ranson *et al*., 2001; Ortelli *et al*., 2003; reviewed in Hemingway *et al.*, 2004; reviewed in Enayati *et al*., 2005). Elevated GST levels in *An. gambiae* were shown to be associated with DDT resistance (Prapanthadara *et al*., 1996; Ranson *et al*., 2001). Ranson *et al*. (2000b) reported that both *cis* and trans-acting factors were involved in the over-expression of the ε-class GSTs in *An. gambiae*.

Implication of GSTe2 involved in detoxification of DDT was only reported in 2003 by Orelli *et al*. Of the four GST proteins (GSTe1, GSTe2, GSTe4 and GSTe8), DDT dehydrochlorinase activity was confined to the recombinant GSTe2 protein (Ortelli *et al*., 2003).

GSTs have also been shown to confer DDT resistance in *Nilaparvata lugens* through protecting tissues from reactive oxygen species damage supplementing other primary resistance mechanisms (Huang *et al*., 1998).

The first cloned GST, PxGSR3 was from the diamondback moth *Plutella xylostella*. Hybridisation of southern blots from methyl parathion-resistant (MPA) strain and susceptible strains showed that resistance was not due to gene amplification (Vontas *et al*., 2001)

1.4.2.2 Caboxylesterase-based resistance

The caboxylesterase-based resistance has been reported from more than thirty insect pests (Hemingway and Karunaratne, 1998). In *Culex* and *Anopheles sp*., it is the primary mechanism for organophosphate insecticide (OP) resistance as well as a secondary mechanism for carbamate resistance (Peiris and Hemingway, 1993). Esterases produce a broad spectrum of resistance in many *Culex* species but in *Anopheles*, this resistance is usually specific to the OP malathion (Hemingway, 1983).

1.4.2.3 P-glycoproteins in invertebrate pest

P-glycoproteins (p-gp) are membrane-spanning proteins from the superfamily of ATP-binding cassettes (ABC transporters) which pump molecules out of cells by an ATP-dependent mechanism (Germann and Chambers, 1998). These proteins have been associated with resistance to a number of drugs, particularly cancer therapy drugs (Juliano and Ling, 1976). Although the normal role of the protein is not clear, what is certain is that p-gp interacts with a large range of chemicals (Germann and Chambers, 1998). It is possible that this system represents the first line of defense to the penetration of xenobiotics into the cell. Preliminary results show that the chemicals such as verapamil that block the action of p-gp do increase insecticide toxicity in *Culex pipiens* and *Cx. quinquefasciatus* (Buss *et al*., 2002).

1.4.2.4 Monooxygenase-based resistance

The P450 enzymes, also referred to as mixed function oxidases (MFOs) or cytochrome (CYP) P450 monooxygenases, are found in virtually all insect tissues (reviewed in Feyereisen, 1999). Originally discovered in rat liver microsomes by Klingenberg in 1958 (reviewed in classic paper as Klingeberg[2], 2003), P450s are a large superfamily of heme proteins which play a crucial role in the biosynthesis of a number of endogenous compounds such as steroid hormones, vitamin D3 and eicosanoids and are also involved in the activation or detoxification of a vast

variety of xenobiotics (Guzov *et al*., 1996; Schuler, 1996; reviewed in Scott, 1999; reviewed in Feyereisen, 1999).

Since this thesis focuses on this form of resistance, it is described in more detail in the next section.

1.5 The cytochrome P450 gene superfamily

Cytochrome P450s are characterised by an intensive absorption band at 450 nm in the presence of carbon monoxide hence the "P" stands for pigment (Omura and Sato, 1964). Synergism studies using piperonyl butoxide (PBO), an inhibitor of cytochrome P450, suggest that P450-mediated detoxification is involved in insecticide resistance (reviewed in Feyereisen *et al*., 1999; Brooke *et al*., 2001). Due to the implications of P450s as a primary route of insecticide detoxification, understanding the mechanisms by which these enzymes are regulated is of prime importance.

1.5.1 P450 classification

The nomenclature system was originally introduced by Nebert and Gonzalez, 1987 and is constantly being revised and updated (Nebert *et al*., 1991, Nelson *et al*., 1993, 1996). This system proposes that all members of a family share >40% identity at the amino acid level whilst members of a subfamily share >55% identity. (Nelson *et al*., 1996). The CYP prefix is used to designate a P450

followed by a number for the family, a letter for the subfamily and a number for the individual gene. P450s use electrons from NAD(P)H to catalyse the activation of molecular oxygen, leading to the regiospecific and stereospecific oxidative attack of structurally diverse chemicals (Werck-Reichhart and Feyereisen, 2000).

1.5.2 P450 characteristic structural features

P450s can be divided into classes depending on how electrons from an NAD(P)H are delivered to the catalytic site (Werck-Reichhart and Feyereisen, 2000). Class I P450 enzymes are found in eukaryotic and bacterial mitochondrial membranes. They require both an FAD-containing reductase and an iron sulphur redoxin as electron donors (Modi *et al*., 1995). This group includes P450cam of *Pseudomonas putida*, the first P450 to be characterized by x-ray crystallography (Omura, 1999). Class II are found in the endoplasmic reticulum of eukaryotes (Weick-Reichhart and Feyereisen, 2000). These proteins only require a single FAD/FMN-containing P450 reductase for transfer of electrons for example cytochrome P450 BM3 of *Bacillus megaterium* (Modi *et al*., 1995). Class III enzymes on the other hand are self-sufficient and require no electron donor while the Class IV enzymes receive electrons directly from an NAD(P)H for example P450nor of *Fusarium oxysporum* (Degtyarenko, 1995). Class I and II P450s from all organisms participate in the detoxification or sometimes the activation of xenobiotics. Class III and IV are considered remains of the most ancestral forms of P450 involved in detoxification of harmful activated oxygen species (Werck-Reichhart and Feyereisen, 2000).

Although the name P450 implies similarity in function, they include a highly diverse array of protein sequences possessing common catalytic chemistry but different metabolic capabilities (Schuler, 1996). The core of the protein is formed by helices, two sets of beta sheets and a meander (Werck-Reichhart and Feyereisen, 2000). The conserved FXXGXXXCXG sequence of the heme-binding decapeptide contains a conserved cysteine that serves as a fifth ligand to the heme iron. This domain is found on the C-terminus of a P450 protein (Fig. 1). On the N-teminus, is the DGXXT domain which is associated with the formation of the oxygen-binding pocket of helix I, needed to stabilize the core structure. Located between these two domains is the conserved EXXR pair which corresponds to the proton transfer groove on the distal side of the heme (Fig. 1).

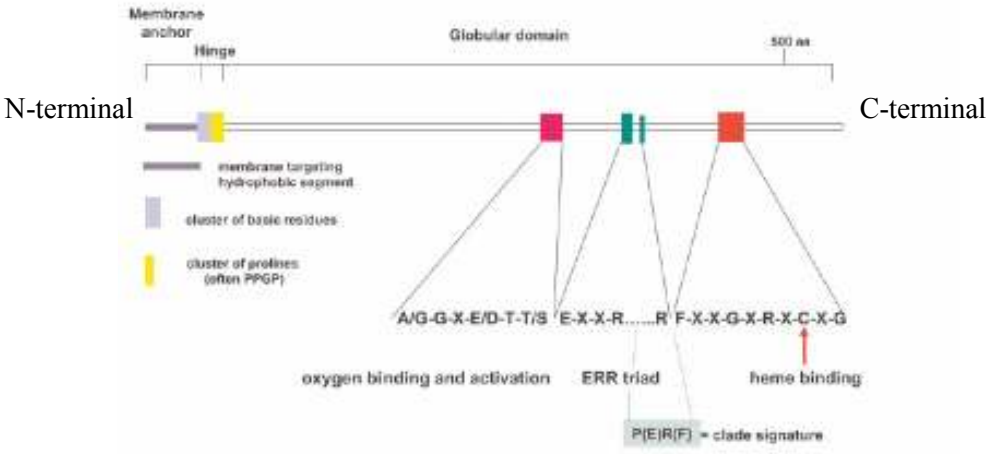


Fig. 1: Conserved structures and sequences in P450 proteins (adapted from Werck-Reichhart *et al*., 2002).

1.5.3 P450 diversity and expression in insects

More than 300 P450s have been identified in insects and are distributed throughout 48 CYP families. These include CYP4, 6, 9, 12, 18, 28, 49 and 301-341 (http://drnelson.utmem.edu/CytochromeP450.html). The CYP6 family is exclusively found in insects and is the most extensively studied group of insect P450s (Liu and Scott, 1998; Winter *et al*., 1999; Kasai *et al*., 1998, Wen and Scott, 2001a; Nikou *et al*., 2003; Rongnoparut *et al*, 2003; Rodpradit *et al*., 2005). Based on phylogenetic analysis by Nelson (1998), CYP6 is closely related to the drug-metabolising CYP3 and the CYP5 family in mammals, CYP30 from clams and CYP9 in insects. The CYP4 family on the other hand, has been shown to be the family with the highest number of insect P450 genes (Scott *et al*., 1994; Adams *et al*., 2000; Ranson *et al*., 2002a,b). The prevalence was based on the assumption that initial insect P450s were recruited within the CYP4 family. Therefore, this made it the favoured gene recruited for new P450 functions in insects (Nelson, 1998).

Examining P450 diversity in insects has revealed numerous genes. For example by using degenerate primers, 17 genes of the CYP4 family were identified from *An. albimanus* (Scott *et al*., 1994), 8 CYP4 from the cotton bollworm *Helicoverpa armigera* (Pittendrigh *et al.,* 1997) and 14 CYP6 and CYP4 P450s from the fruitfly *Ceratitis capitata* (Danielson *et al*., 1999). Sequencing of the *Drosophila melanogaster* genome has uncovered a total of 90 P450s including four pseudogenes (Adams *et al*., 2000). In *An. gambiae*, 111 P450s including seven

pseudogenes have been identified (Ranson *et al.,* 2002a, b). A recent study on *An. minimus* has led to the isolation of three CYP6 P450s (Rongnoparut *et al*., 2003; Rodpradit *et al*., 2005). Other P450s have been isolated from *Musca domestica* (Feyereisen *et al*., 1989; Liu and Scott, 1998; Guzov *et al*., 1998; Kasai and Scott, 2000), *Blattella germanica* (Wen and Scott, 2001a) *Blaberus discoidalis* (Bradfield *et al*., 1991), *Locusta migratoria* (Winter *et al*., 1999) and *Cx. quinquifasciatus* (Kasai *et al*., 1998).

Expression of P450s has been shown to be developmentally regulated (Wen and Scott, 2001b; Pridgeon *et al*., 2003). CYP6L1 was examined in life stages of *B. germanica* and expression was only detected in the adults (Wen and Scott, 2001b). In the a pyrethroid resistant stain of *B. germanica*, CYP4G19 expression was very low in the eggs, increased in the nymps and was maximum in the adults (Pridgeon et al., 2003). In certain instances, tissue specific expression has been observed (Wen and Scott, 2001b). For example, CYP6L1 mRNA was expressed exclusively in the testes and accessory glands of male cockroaches (Wen and Scott, 2001b).

Expression of P450s may also show sex specificity. Specifically, male expression has been reported in *B. germanica* CYP6L1 (Wen and Scott, 2001b) and CYP312a1 in *D. melanogaster* (Kasai and Tomita, 2003). CYP6Z1 in *An. gambiae* (Nikou *et al*., 2003) showed increased expression in males compared to females of the same strain. For example the susceptible strain showed an 11.5 fold

increase in males while the resistant males showed a 30 fold increase compared to resistant females.

1.5.4 P450 catalysed reactions

P450s catalyse many types of reactions including oxygenations, dehalogenations, dealkylations, deaminations, dehydrogenations and isomerations (Nebert and Gonzalez, 1987). The enzyme reactions are based on activation of molecular oxygen with insertion of one of its atoms into the substrate and reduction of the other to form water (Guengerich, 1991; Porter and Coon, 1991). The generic equation for the cytochrome P450 catalysed MFO reaction is expressed as follows:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Where RH and ROH represent the substrate and oxidised product respectively.

This reaction is best represented as a cyclic reaction showing the interactions that occur between cytochrome P450 and the NADPH cytochrome P450 reductase (Guengerich, 1991) (Fig. 2). Briefly, the reaction cycle is initiated by the binding of the substrate (RH) to the ferric form of the enzyme to form an enzyme-substrate complex (ferric complex) (Step I). This is followed by the reduction of the ferric complex by an electron transferred from NADPH via NADPH-cytochrome P450 reductase. Molecular oxygen binds to the reduced complex to form an enzyme-oxygen-substrate complex followed by the transfer of a second electron from NADPH via NADPH-cytochrome P450 reductase or from

cytochrome b_5 (Step II). A further addition of a second proton results in splitting of the oxygen-oxygen bond with one atom of oxygen being released as water. The retained oxygen atom is transferred to the substrate, giving rise to an oxidised product (ROH), which is released and a ferric form of the enzyme is once more generated and the cycle begins again (Step III). In mitochondria, electrons are transferred from NADPH by redoxin reductase to redoxin and then to the enzyme.

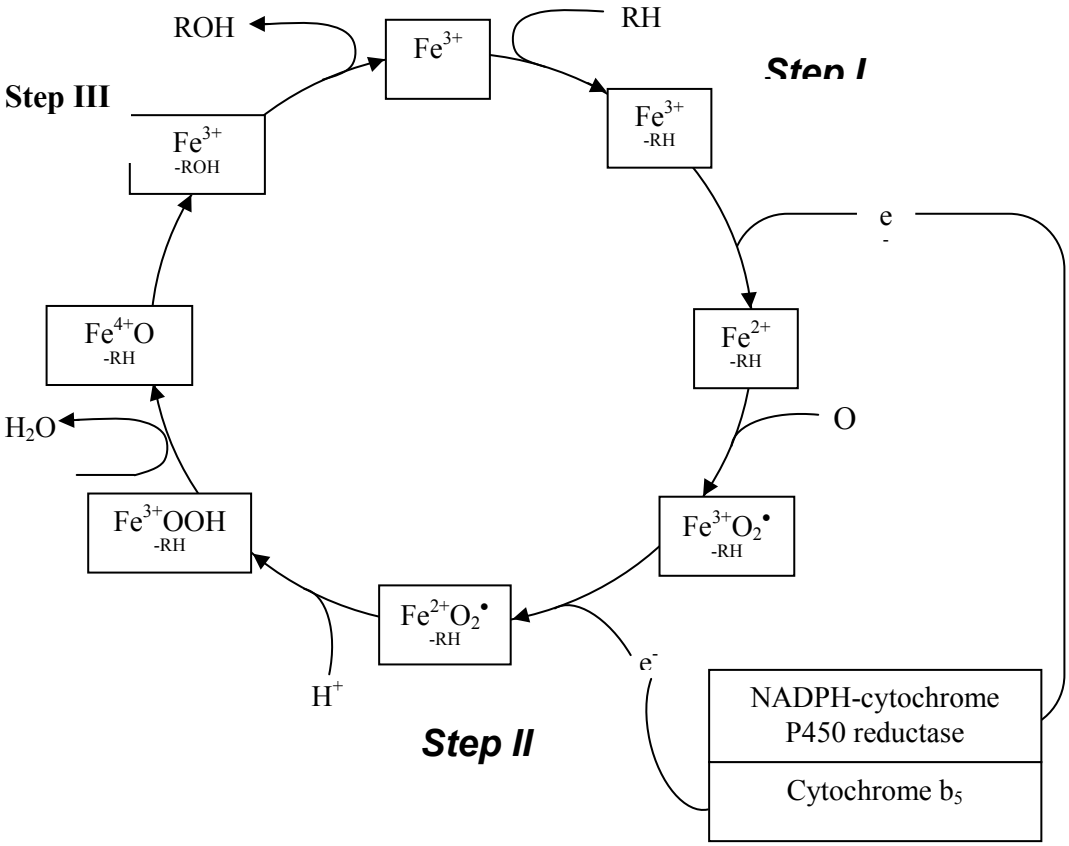


Fig. 2: Mechanism of cytochrome P450-dependent hydroxylation. RH is the substrate (Guengerich, 1991).

1.5.5 Isolation of individual insect P450s

A number of strategies can be used to isolate genes from any multigene family. Sequencing the entire genome is a systematic approach to isolate P450 genes (Adams *et al*., 2000; Ranson *et al*., 2002a,b) but the method is currently not feasible for most insects, as the entire genome has to be available. Purification of P450s from insecticide resistant insects and the development of antiserum, which can then be used to screen a cDNA expression library, have also been employed (Feyereisen *et al*., 1989). The drawback with this method is that related P450s can be isolated (Scott and Wen, 2001). Due to the similarity between P450s in organisms, a previously identified P450 can be used as probe to isolate new P450 genes in a related organism (Xiao-Ping and Hobbs, 1995). In order to isolate P450s with a specific function, the use of differential or subtractive screening can be employed (Bradfield *et al*., 1991).

Although different approaches have been successfully employed to isolate individual insect P450s (Snyder *et al*., 1996; reviewed in Scott, 1999; Scott and Wen, 2001), few match the speed and efficiency of PCR-based method that employ degenerate primers. PCR is a powerful tool for obtaining short DNA sequences. This method is based on similarities in the deduced amino acid sequences of different P450 families (Snyder *et al*., 1996). In one technique, the forward primer designed, targets sequences from the heme-binding decapeptide whilst the vector primers (in the case of library screening) or oligo(dT) primers (for cDNA) are used as reverse primers. In another strategy, a reverse degenerate primer is designed within the region surrounding the conserved cysteine and a

forward primer targets the conserved region of the helix I (Snyder *et al*., 1996). PCR products obtained using these methods can then be used to screen libraries (Xiao-Ping and Hobbs, 1995).

1.5.6 Role of P450s in insecticide resistance

The cytochrome P450 enzymes metabolise insecticides resulting either in bioactivation or in detoxification, the latter process being enhanced in many strains with metabolic resistance to insecticides (Lu and Coon, 1968; Nebert and Gonzalez, 1987). The over-expression of the P450s in pyrethroid-resistant insects and their possible role in enhanced metabolic detoxification of pyrethoid insecticides is well documented (Lui and Scott, 1998; Nikou *et al*., 2003; Pridgeon *et al*., 2003; Wen and Scott, 2001a, b; Rodpradit *et al*., 2005). For example, the over-expression of CYP6D1 and CYP9A1 was found to contribute to resistance to pesticides in *M. domestica* (Tomita and Scott, 1995) and the tobacco budworm, *Heliothis virescens* (Rose *et al*., 1997) respectively. Although the increase in resistance has been thought to occur as a result of the over-expression of these enzyme proteins, studies have shown that when levels normalise, resistance is not necessarily lost (Bergé, *et al*., 1998).

Enhanced transcriptional expression is possibly one of the underlying mechanism of CYP over-expression that confers insecticide resistance (Rose *et al*., 1995; Liu and Scott, 1998). Darbon *et al*., (2002) reported that a resistant allele of CYP6Gl in *D. melanogaster* contains an *Accord* transposable element, which is responsible

for the upregulation of transcription in this gene. In *An. gambiae*, the increase in expression of an adult-specific CYP6Z1 gene is not due to gene amplification, but thought to result from a mutation in the promoter or regulatory element that controls expression in CYP6Z1 (Nikou *et al*., 2003).

In the public health markets pyrethroids are used for indoor residual house spraying and insecticide treated bednets and curtains. This is due to their very low toxicity to humans and rapid killing effect on insects. Worryingly, several studies have demonstrated elevated P450 monooxygenase activity in insecticide resistant *Anopheles* species (Vulule *et al*., 1999; Brooke *et al*., 2001). Since pyrethroids are the main insecticide promoted for the control of malaria vectors, it is important that their efficacy is maintained as long as possible. The identification of the particular P450 gene(s) associated with resistance is important in understanding of their role in pyrethroid resistance. In addition, with a comprehensive picture of P450 genes found in an organism, studies focussing on the molecular basis of insecticide resistance and factors that govern their evolution will be possible.

1.6 Rationale of the study

1.6.1 Malaria situation in South Africa

Malaria occurs in limited areas in South Africa, mainly at low altitude (below 1000 m) in Limpopo, Mpumalanga and northeastern Kwazulu-Natal provinces (Department of Health, 1996). Limited focal transmission may occasionally occur

in North West and Northern Cape provinces along the Molopo and Orange rivers (Department of Health, 1996). Malaria is distinctly seasonal in South Africa with the highest risk being during the wet summer months (October-May) (Department of Health, 1995). The number of cases and deaths from malaria from 1971 to 2004 are given in Fig. 3. The increase in cases is attributed to the replacement of DDT with synthetic pyrethroids in 1996 leading to the re-surgence of *An. funestus* pyrethroid resistant populations (Hargreaves *et al*., 2000). Biochemical and bioassays carried out by Brooke *et al*. (2001) implicated monooxygenase detoxification as the major resistance mechanism in resistant *An. funestus* mosquitoes from South Africa.

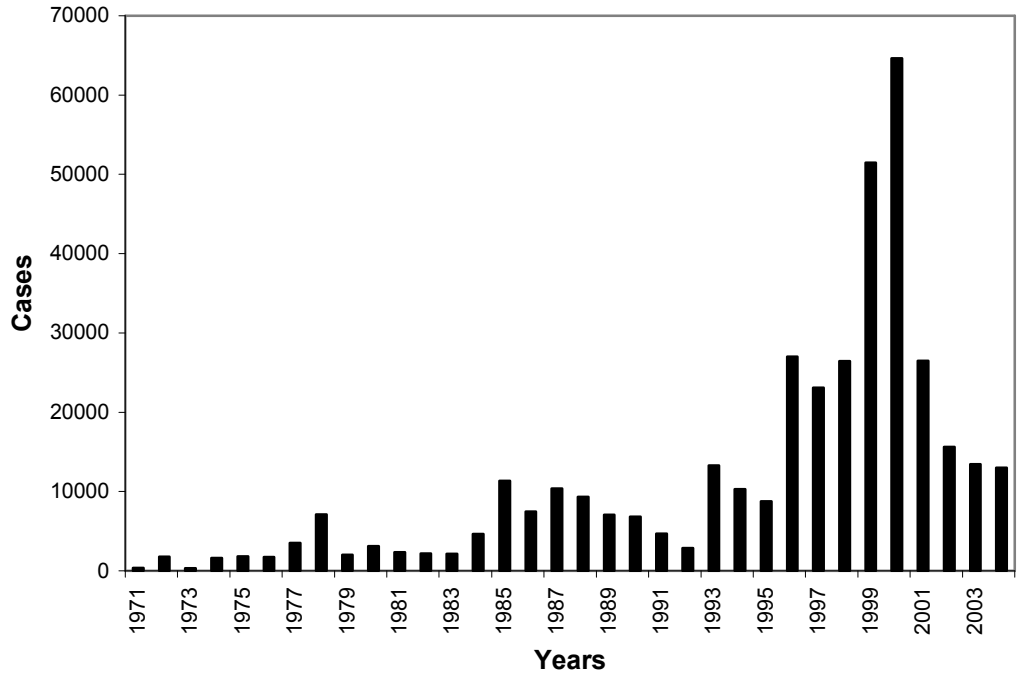


Fig. 3: Total malaria cases for South Africa (1971 – 2004) (source: Department of Health, unpublished records).

Demonstration of resistance to the pyrethroid insecticide, deltamethrin forced the national malaria control programme to change their control policy by reverting back to the use of DDT (Hargreaves *et al.,* 2000; 2003). As a result, spraying of traditional houses with DDT and western-style houses with pyrethroids has in effect eliminated *An. funestus* and reduced the number of malaria cases (Hargreaves *et al*., 2003). Understanding the mechanism involved in insecticide resistance is important in order to develop more precise methods of measuring this phenotype, which will assist in future decisions on insecticide usage made by vector control authorities.

1.7 Aim and objectives

1.7.1 Aim

Studying the mechanism of metabolic resistance in *An. funestus* was hindered by the fact that there was no published information available on P450 genes in this species. This project aimed at obtaining information on P450 gene families in *An. funestus* and investigating their association with pyrethroid resistant and susceptible strains of this species.

1.7.2 Specific objectives

The specific objectives were as follows:

1. To isolate and sequence multiple P450 genes from 3 families (CYP4, CYP6 and CYP9) in *An. funestus*.
2. To identify the family of P450 genes over-expressed in pyrethroid resistant *An. funestus*.
3. To identify specific gene(s) within the P450 family that is over-expressed in the resistant compared to the susceptible strain of *An. funestus*.
4. To identify by semi-quantitative reverse transcriptase PCR (RT-PCR) the expression profile of the over-expressed gene in the resistant compared to the susceptible strain of *An. funestus*.
5. To confirm P450 gene expression using quantitative Real-time PCR.
6. To construct a cDNA library from pyrethroid resistant *An. funestus*.
7. To optimise conditions for cDNA library screening using P450 probes.

CHAPTER 2

MATERIALS AND METHODS

The present chapter focuses on biological material and modification of standard procedures that were carried out to obtain results on *An. funestus*. Detailed description of the standard procedures containing the recipes is presented in Appendix A.

2.1 Biological material

Two laboratory colonies of *An. funestus* have been established at the Vector Control Reference Unit of the NICD in Johannesburg, South Africa. Both colonies originate from southern African. The first colony originates from Mozambique and contains the pyrethroid resistant phenotype while the second colony originates from Angola and was found to be susceptible to both deltamethrin and DDT using standard WHO susceptibility assays (Hunt *et al*., 2005). These are the only colonies of *An. funestus* currently in existence worldwide. The resistant strain called FUMOZ-R was selected initially on 0.1% lambda-cyhalothrin and then on increasing dosages of permethrin over successive generations using the methods described in Hunt *et al.,* (2005). The resistant strain is currently exposed at each generation to 1.5% permethrin (double the WHO discriminating dose). The susceptible strain, FANG shows 100% mortality when exposed to 0.75% permethrin for 1 hr. The two colonies are maintained at a

temperature of 25 ^{o}C with 80% relative humidity, 12 h day/night and 45 min dusk/dawn lighting cycle.

2.2 Preparation of samples

Total RNA was isolated from 3-day-old adult resistant *An. funestus* (FUMOZ-R) without being exposed to insecticide. Mosquitoes were immobilised briefly at –20 ^{o}C and homogenised in Tri-reagent (Sigma, cat. no. T9424) using a polystyrene pestle as described by the manufacturers instructions with modifications. For every 3 mosquitoes, 200 µl of Tri-reagent was used. Chloroform (0.2 vol) was added to the mixture incubated at room temperature for 5 min and centrifuged (11,269 x g, 15 min, 4 ^{o}C). The supernatant fraction was precipitated using 2 vol of room temperature isopropanol and incubated for 10 min. The RNA precipitate was recovered by centrifugation (7,826 x g, 10 min, 4 ^{o}C) and the pellet rinsed with 3 vol of 75% ethanol and re-centrifuged (4,402 x g, 5 min, 4 ^{o}C). The pellet was re-suspended in 20 µl of 0.01% (v/v) diethyl pyrocarbonate (DEPC) treated water. The RNA integrity was determined on a 1.2% (w/v) agarose formaldehyde gel and its concentration quantified using a GeneQuant *pro* Spectrophotometer (Amersham Biosciences).

2.3 Reverse Transcriptase (RT) PCR

In order to isolate P450s from *An. funestus*, RT-PCR method was carried out. One µg of the total RNA was primed with 500 µg oligo(dT) adaptor (5'-GACTCGAGTCGACATCGA-3') and reverse transcribed using Superscript III

(Invitrogen, cat. no. 18080-044) according to manufacturer's instructions. RT efficiency was however not calculated. PCR was carried out using 50 ng of cDNA synthesised from Superscript III. Other PCR components consisted of 10 X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 10 μM of each degenerate CYP4 primer (Forward, 5'-GAG GAY TTC ATG TTC GAR GGC ACG AYA-3'; Reverse, 5'-CTG CCG ATR CAG TTC GBG GCC GCW GAA BGG-3'), CYP6 primer (Forward, 5'-TCW SIC TGT ACG AGC TKG C-3'; Reverse, 5'-GGI CCC TCG CCG AAC GG-3') or CYP9 primer (Forward, 5'-TTY ACC GGC AGC AAR ATG CG-3'; Reverse, 5'-AGY GTY TCC GAY ACR ACC ATR TC-3'), 10 mM dNTPs, 1.5 mM $MgCl_2$ and 0.5 units Takara^{TM} Taq polymerase (Takara Bio Inc.).

Thermocycling conditions consisted of an initial denaturation step at 94 ^{o}C for 15 min, followed by 35 cycles of 94 ^{o}C for 1 min, 50 ^{o}C for 30 s and 72 ^{o}C for 2 min. A final auto extension of 72 ^{o}C for 10 min completed the reaction. Amplification products were analysed by electrophoresis alongside 1 kb DNA size marker (Invitrogen, cat. no. 10787-018) on 1% (w/v) agarose gel in 1X TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8). Gels were stained with Ethidium Bromide (EtBr) (10 mg/ml). The amplified bands were visualised by illumination using a Hoefer Macrovue UV-25 UV-illuminator (Amersham biosciences, UK) and photographed.

2.4 Cloning of PCR products

Amplified PCR products were purified prior to cloning. Elution of DNA was carried out using QIAquick™ PCR purification kit (Qiagen, cat. no. 28106) according to manufacturer's protocol. Purified PCR products of CYP4, CYP6 and CYP9 were ligated into pGEM® -T easy vector (Promega**,** cat. no. A1360) and transformed using *Escherichia coli* (*E. coli*) strains JM109 bacterial cells (Promega, cat no. L2001). To minimise repeated sequencing of identical clones, the selected cloned products were further analysed by SSCP as described by Koekemoer *et al*. (1999). Silver staining was carried out according to Budowle *et al*. (1991). Recombinant plasmids from cloned products were extracted using Qiagen® plasmid minikit (Qiagen, cat. no. 12125) following manufacturer's instructions and sequenced. Sequencing was carried out using the ABI PRISM ® BigDye TM Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) according to manufacturer's instructions.

2.5 Sequence analysis and Phylogenetic analysis

Sequences were searched against the *An. gambiae* P450 database using the BLAST-X algorithm at the P450 analysis website (http://p450.antibes.inra.fr/). Sequences of clones that did not have conserved domains characteristic of P450 after BLAST were excluded from further analysis. The vector and primer sequences were trimmed from the scf files using the Vecscreen programme (www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Resultant sequences were

analysed using DNASTAR™ (Lasergene). *Anopheles funestus* genomic sequences of CYP4, 6 and 9 classes were included in the analysis. With these sequences, intron-exon organization in *An. funestus* P450s was determined using the canonical GT/AG rule (Mount, 1982) and compared with that of *An. gambiae*. The relationship between *An. funestus* and *An. gambiae* P450s was determined using phylogenetic analysis. Sequence alignment was performed by ClustalW (BIOEDIT programme (Hall, 1999) and tree construction carried out using MEGA 2.1 (Kumar *et al.*, 2001).

2.6 Construction and screening of *An. funestus* cDNA library

A λTriplEx2 cDNA library was constructed using the SMART™ cDNA library construction kit user manual (BD Biosciences, cat no. K 1051-1). Fourty six μg/μl total RNA was isolated from 30 adult pyrethroid resistant (FUMOZ-R) *An. funestus*. Thirty three μg/μl poly $(A)^+$ mRNA was purified from total RNA using GeneElute mRNA miniprep kit (Sigma, cat. no. mrn 10). The library was packaged in Packagene® Lambda DNA packaging system (Promega, cat. no K3154) according to manufacturer's instructions. Optimisation of cDNA library screening was carried out as described by Sambrook *et al*., (1989) using P450 Digoxigenin (DIG) labelled probes and *E. coli* XL1-blue cells.

2.7 Expression of P450 genes in a pyrethroid resistant *An. funestus*

2.7.1 Blot analysis

Dot blot analysis was carried out to identify the class of P450 that was over-expressed in the resistant strain of *An. funestus*, FUMOZ-R. RNA was prepared from 3-day old FUMOZ-R and the susceptible strain, FANG. Serial dilutions of RNA (from 25 μg to 0.049 μg) were spotted onto a nylon membrane. The membranes were UV crosslinked and hybridised. The same method was used to determine the over-expressed gene. For this, serial dilutions were carried out from 50 μg to 0.098 μg.

Expression of a specific P450 class was further carried out. To carry this out, an individual gene within the class from which a signal was detected was used to probe RNA on a membrane. Increase in signal intensity between FUMOZ-R and FANG was monitored at various RNA concentrations, which were serially diluted. The expression fold of the identified gene was compared in the two mosquito strains. The dot blots were repeated four times with independent RNA preparations.

The over-expression of CYP6P9 was validated using northern blot analysis. CYP6P9 probe was used to probe RNA on the membrane. RNA (5 μg) was electrophoresed on a 1.2 % formaldehyde agarose gel and transferred by capillary action as described by Sambrook *et al*., (1989).

2.7.2 Semi-quantitative RT-PCR

To determine the expression profile of CYP6P9, semi-quantitative RT-PCR was carried out using cDNAs prepared from eggs, larvae, pupae and 3-day-old adult males and females of FUMOZ-R and FANG mosquitoes. Specific oligonucleotide primer pairs for CYP6P9 were designed using Beacon Designer 3.0 software (PREMIER Biosoft International) (Fig. 4). One primer pair RTF1-F (5'-GTC GTG TGC CGT CTG TTG-3') and RTF1-R (5'-ATG TCG CTT CTT CAC TTC CTC-3'). The second primer pair RTF2-F (5'-AGA ATC GTT GAG TCG TGT G-3') and RTF2-R (5'-TGT TGA ATG GCG TGA AAC-3'). The RTF1 and RTF2 primer pairs amplified product sizes of 169 and 104 bp respectively.



Fig. 4: Primer location for RTF1 and RTF2 primer pairs. Red: RTF2 primer pair; Bold: RTF1 primer pair.

The rsp7 gene was used to normalise transcript levels. Rsp7 primer Qrsp7B-F (5'-TTACTG CTG TGT ACG ATG CC-3') and Qrsp7B-R (5'-GAT GGT GGT CTG CTG GTT C-3') amplified a product size of 135 bp. PCR was carried out as described in Appendix A except for the primer concentration of 2.5 μM and template concentration of 50 ng. PCR cycling condition was as that used to screen inserts in cloned degenerate PCR products. Band intensities were compared in the PCR products after normalisation. PCR reactions were repeated three times with cDNAs prepared from independent RNA extractions.

2.7.3 Quantitative real time (qRT) PCR

Quantitative real time (qRT) PCR was used to validate the results obtained using semi-quantitative and blot analysis. Real time PCR is based on the basic principle of the measurement of a fluorescent signal which is proportional to the amount of the amplified product (Peters *et al*., 2004; Qiagen, 2004). Therefore, the amplification cycle at which fluorescence rises above an arbitrary threshold cycle (C_T) is inversely proportional to the logarithm of the initial copy number in a sample. Real time PCR was carried out using SYBR green chemistry on MyiQ^{TM} Single-color real time PCR detection system (Biorad Laboratories, Inc., USA) and the hybridisation probes chemistry on a LightCycler rapid thermal cycler system 1.0 (Roche Diagnostics GmbH, Germany).

To examine developmental expression of CYP6P9 gene, cDNAs were prepared from DNAse treated RNA. Ten third instar larva and ten pupa from both

FUMOZ-R and FANG were used and the methodology is presented in Chapter 2. Expression was compared in males and females of FUMOZ-R and FANG using cDNAs prepared from DNAse treated RNA of ten individuals. Time-course expression of CYP6P9 gene, was examined using cDNAs prepared from ten 3-day old and ten 14-day old males and females of FUMOZ-R.

2.7.3.1 SYBR Green 1 chemistry

SYBR Green I is an intercalating dye which binds to double stranded DNA and results into an increase in fluorescence as the amount of PCR product increases (Peters *et al*., 2004). The products detected by SYBR Green I can be observed by generating a melt curve at the end of the PCR amplification cycles.

PCR conditions were optimised for both CYP6P9 and rsp7 primers. A gradient PCR was carried out using CYP6P9 and rsp7 plasmids with annealing temperatures ranging between 50 ^{o}C to 60 ^{o}C and a range of template concentrations. An annealing temperature of 55 ^{o}C worked well for the RTF1 and the Qrsp7B primer pairs. RTF2 primer was resulted in primer dimers and was omitted in subsequent experiments.

Absolute quantification was carried out by generating standard curves from plasmid dilutions of CYP6P9 and rsp7 genes with concentrations ranging from 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg and 10 fg. Plasmid reactions were set up in duplicate in clear PCR tube strips (Biorad laboratories, cat. no. TBS 0201).

Similarly, PCR reactions for the cDNAs were set up in duplicate from two independent RNA extractions for larva and pupa and three for adults from both FUMOZ-R and FANG. Amplification mix (20 µl) consisted of 10 µl 2 X iQ™ SYBR® Green Supermix (Biorad Laboratories, Inc. cat. no. 170-8880), 250 nM of each primer (RTF1-F and RTF1-R or Qrsp7B-F and Qrsp7B-R), 100 ng of cDNA template and water. The tubes were sealed using ultra clear caps (MJ research, cat. no. TCS-0803). The tubes were briefly centrifuged (700 x g for 5 s) and put in the real time machine.

PCR conditions consisted of an initial denaturation and hot start DNA polymerase activation at 95 ^{o}C for 10 min, followed by amplification of 40 cycles of 95 ^{o}C for 30 s, 55 ^{o}C for 30 s and 72 ^{o}C for 30 s. After 40 cycles of PCR, samples were run for the dissociation protocol. Generation of melt curve was carried out at 55 ^{o}C with increments of 0.5 ^{o}C every 1 s. Amplification products were run on a 1.5% agarose gel stained using EtBr to confirm specificity of the products. The cDNA experiments were repeated using rsp7 gene primers to normalise the data obtained using CYP6P9 primers.

2.7.3.2 Hybridisation probes chemistry

This detection method relies on the fluorescence resonance energy transfer (FRET) principle that involves two oligonucleotide probes (Kurupati *et al.*, 2004). One probe is labelled with a donor fluorochrome (fluorescein) at the 3' end. The second probe is labelled with an acceptor dye such as LightCycler Red (LC Red)

640 at the 5' end and phosphorylated at the 3’ end (Roche Diagnostics GmbH, Germany). The two probes bind to an internal sequence of the amplified fragment during the annealing cycle. During FRET, fluorescein is excited and part of this excitation energy is transferred to the LC Red dye. The LC red dye emits fluorescence which is measured by the LightCycler instrument. As a result of their sequence specific detection, a signal can only be generated when the correct amplicon is amplified.

LightCycler FRET hybridization probes were designed within the target regions amplified by either RTF1 or Qrsp7B primer pairs for CYP6P9 and rsp7 genes respectively. The CYP6P9 probes were named target probe 1 (GCA AAT TCC GGT TCA CGC CAT-fluorescein) and target probe 2 (LC Red 640-AAC ATG ATC CTG AGC ACT ATC CCG ATC CAG-phosphate). The rsp7 probes were named reference probe 1 (TTC CCC GCG GAG GTT GT-fluorescein) and reference probe 2 (LC Red-GCA AGC GTA TCC GCG TGA AGC T-phosphate).

Real time PCR was carried out using LightCycler® FastStart DNA MasterPLUS HybProbe (Roche Diagnostics cat no. 03 515 575 001) according to manufacturer’s instructions. PCR reaction master mix consisting of 250 nM each either of RTF1 or Qrsp7B primer pairs, 400 nM each of the specific probes, 4 µl of hybridisation mastermix, and water was prepared. Depending on the total number of reactions, the Lightcycler glass capillaries (Roche Diagnostics cat. no. 11 909 339 001) were placed in pre-cooled centrifuge adaptors. The master mix (12 µl) was aliquoted into the capillaries and 8 µl (250 ng) cDNA samples added

to the respective capillaries. For the standards 19 µl of the master mix and 1µl was added to the respective capillaries. The capillaries were sealed and centrifuged at 700 x g for 5 s. The capillaries containing the samples were transferred to the Lightcycler sample carousel in placed into the Lightcycler instrument (Roche Diagnostics GmbH, Germany).

The PCR LightCycler reaction was carried out with an initial denaturation at 95 °C for 10 min followed by 40 cycles of amplification at 95 °C for 0 s, 55 °C for 10 s and 72 °C for 10 s with a single transition rate of 20 °C/s and a single fluorescence acquisition at 55 °C. After completion of amplification, a melt curve was generated by holding the reaction at 95 °C for 0 s. This was followed by 45 °C for 30 s then slowly heating at a transition rate of 0.1 °C/s to 95 °C with continuous fluorescence acquisition. Finally, the reaction was brought to cool at 40 °C. The PCR products of the cDNAs were isolated from the capillaries at the end of each run and were visualised by electrophoresis on 1.5% agarose gels stained using EtBr.

2.7.4 Quantitative analysis of real time PCR (qRT) data

Results obtained using the MyiQ^{TM} Single-color real time PCR detection system (Biorad Laboratories Inc. USA) were analysed using MyiQ software version (Biorad). Similarly, the results that were obtained using the Lightcycler rapid thermal cycler system (Roche diagnostics Ltd,) were analysed using the

Lightcycler software version 3 using the second derivatives method. For both analysis, quantification was based on threshold cycle (C_T) value or crossing points (C_P), which is inversely proportional to the logarithm (log) of the initial copy number.

A linear relationship was obtained by plotting the C_T or C_P value against the log of copy number obtained with each of the plasmids. The equation of the line that best fits the data was determined by regression analysis using MyiQ software or the Lightcycler™ version 3 software. The data from CYP6P9 and rsp7 plasmid and were cDNA converted to copy number as described by Paton *et al*., (2000). The standard curves were used to interpolate the copy numbers of the cDNAs,. The mean copy number and standard deviation from CYP6P9 and rsp7 gene were calculated for the cDNAs. The CYP6P9 copy number for each cDNA sample was normalised using its respective rsp7 copy number. The copy number ratio between CYP6P9 and rsp7 expression was determined for each independent cDNA. Statistical analysis was carried out on each independent normalised cDNA using student t-test at 95% confidence interval. The graphs were drawn using the normalised mean.

CHAPTER 3

ISOLATION OF P450 GENES FROM A PYRETHROID RESISTANT COLONY OF *ANOPHELES FUNESTUS*

3.1 INTRODUCTION

P450 genes in *Anopheles* have been studied using nucleotide and derived amino acid sequences. In a number of cases, the genes have been mapped to specific chromosome loci (Ranson *et al*., 2002a) and in others, the mechanisms of gene expression studied (Nikou *et al.,* 2003). Although the interaction of insecticides with P450 enzymes has been studied, much more remains poorly understood.

In pursuit of the present objective of trying to identify P450's in *An. funestus*, cloning and sequencing of these genes were carried out. These were important first steps towards establishing the diversity of P450 enzymes in this species. The focus was on CYP4, CYP6 and CYP9 gene families because a number of P450 genes associated with insecticide resistance have been shown to belong to these families (Feyereisen *et al.,* 1989; Rose *et al*., 1997; Pridgeon *et al*., 2003; Nikou *et al*., 2003). An overview of the literature is presented in Chapter 1.

3.2 MATERIALS AND METHODS

Degenerate primers corresponding to the conserved regions of CYP4, CYP6 and CYP9 P450 families of *An. gambiae* were used to amplify cDNAs from *An.*

funestus. The PCR products were sub-cloned into pGEM® -T easy vector and sequenced. Intron-exon organisation was established in partial *An. funestus* P450 genomic sequences. This was carried out using BLASTX algorithm search on *An. gambiae* amino acid full-length P450 sequences (http://p450.antibes.inra.fr/) and Mapdraw using DNASTAR™ (Lasergene) programme. Figure 5 shows the amplified regions of *An. funestus* clones on a referenced P450 gene.

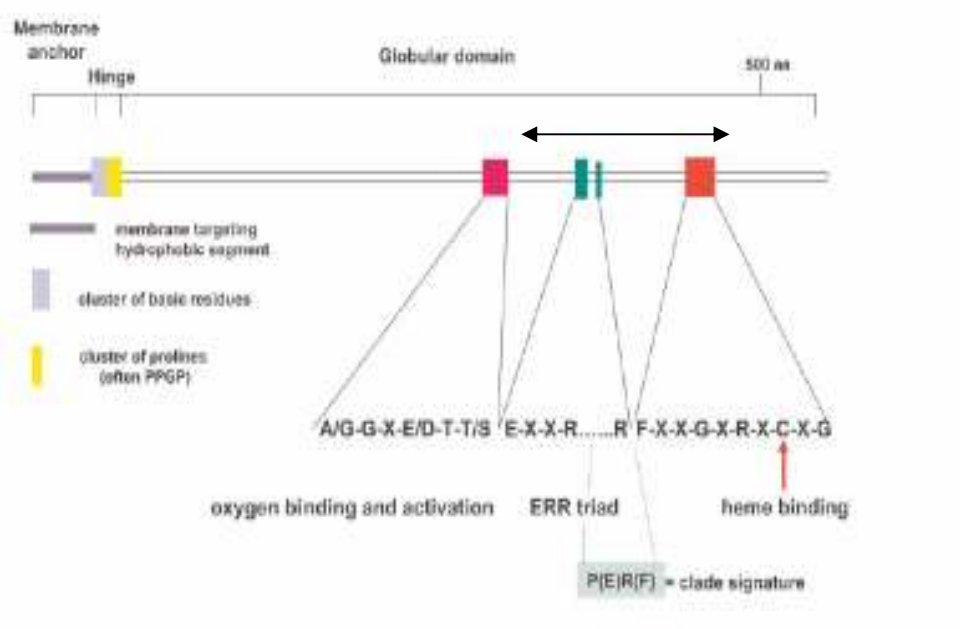


Fig. 5: Reference P450 gene (adapted from Werck-Reichhart *et al*., 2002). The arrow shows the amplified regions of *An. funestus* clones.

The sizes and positions of introns were compared with those of *An. gambiae*. Sequences were analysed and phylogenetic analysis established between *An. funestus* and *An. gambiae* P450s. Details of the methodology are presented in Chapter 2. Using degenerate PCR method, *An. funestus* genomic DNA was amplified, cloned and sequenced (Amenya *et al*., 2005). Sequence data of the

genomic DNA that were used to annotate intron-exon position in *An. funestus* P450 were kindly provided by Hilary Ranson (Liverpool School of Tropical Medicine, UK).

3.3 RESULTS

The results of this chapter have been published in *DNA Sequence*. A copy of the article is attached in Appendix D. What follows is a detailed description of the results and discussion obtained using *An. funestus* cDNA. The results include annotations of the genomic sequences to locate intron-exon position in *An. funestus* P450s.

3.3.1 Total RNA extraction and Reverse Transcriptase (RT) PCR

Total RNA was isolated from 3-day old male and female of *An. funestus*. The RNA was electrophoresed on a 1.2% formaldehyde agarose gel and the separation of the 28S and 18S ribosomal bands showed that it was intact (Fig. 6). After quantification of the RNA, the level of purity as determined by the OD ratio of 260/280 nm was found to be between 1.8 and 2.0. The total RNA was used in RT-PCR using degenerate CYP4, CYP6 and CYP9 primers. Fragment sizes were estimated to be 450, 375 and 750 bp for CYP4, CYP6 and CYP9 genes respectively (Fig. 7). The PCR products were ligated into pGEM® -T easy vector and transformed using JM109 cells. Recombinant colonies were determined by their white colour on the LB agar Ampicillin-treated plates compared to the non-

recombinant ones which were blue in colour. The positive clones were screened for inserts using SP6 and T7 universal primers.

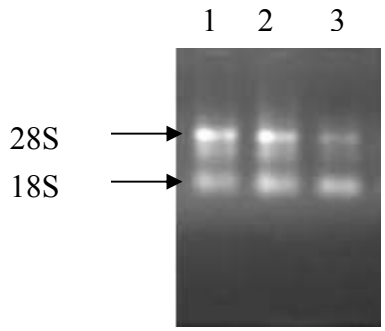


Fig. 6: Denatured agarose gel electrophoresis of total RNA from *An. funestus*. Lanes 1, 2 and 3: Independent RNAs (5 μg) loaded on a 1.2% formaldehyde agarose gel.

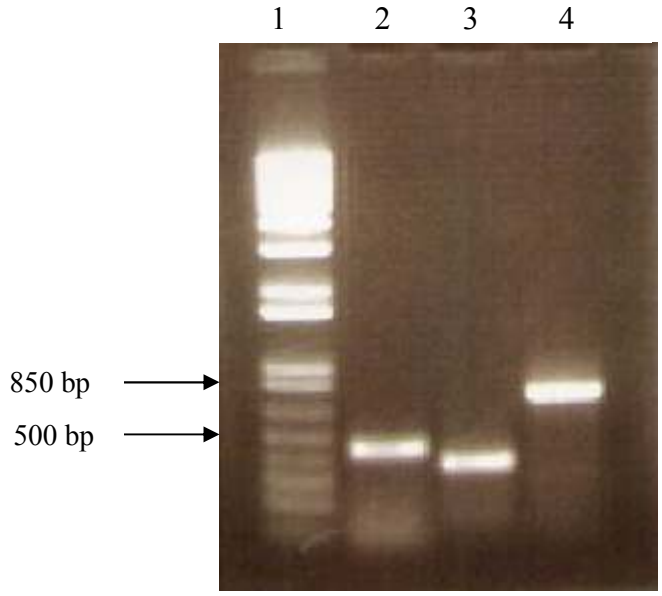


Fig. 7: RT-PCR products from degenerate CYP4, 6 and 9 primers. Lane1: 1 kb plus DNA size marker (Invitrogen), Lane 2: CYP4, Lane 3: CYP6, Lane 4: CYP9.

3.3.2 Screening of clones (transcripts) using Single Strand Conformation Polymorphism (SSCP)

Degenerate primers were used to isolate multiple genes within each family. In order to differentiate between clones with the same gene insert from those with different gene inserts, the clones were screened. The screening was based on the principle of conformational changes of single-stranded DNA molecules migrating in a neutral polyacrylamide gel. Single stranded DNA renature in sequence specific conformations that influences their mobility in a non-denaturing polyacrylamide gel thus detecting DNA polymorphisms and mutations (Orita *et al.*, 1989). The various migration patterns obtained after electrophoresis on various CYP4 clones are shown in Fig. 8. A total of fourteen conformational patterns were detected. Eleven of these had a frequency of 7%, two had a frequency of 14% and one had a frequency of 21%. Clones with different conformational patterns were selected for sequencing. Appendix B shows the clones that were identified as P450s after sequencing.

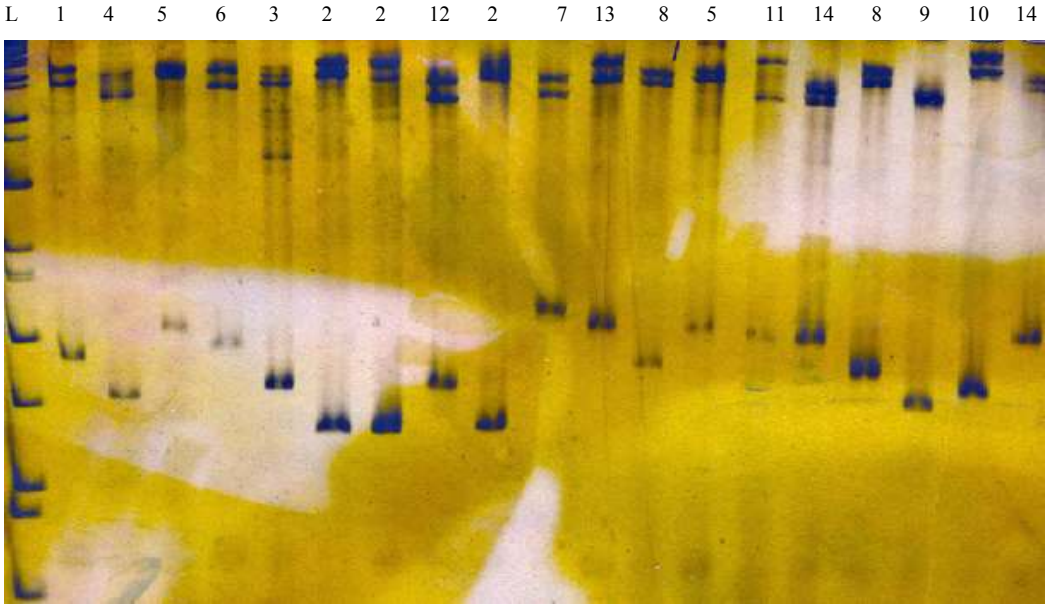


Fig. 8: SSCP gel of amplified inserts of CYP4 clones. L: 1 kb plus ladder (Invitrogen); 1-14: the various conformation patterns obtained from the clones. Lanes with the same patterns were designated the same numerical numbers.

3.3.3 Sequence analysis

Sequencing of screened clones yielded partial P450 gene fragments when blasted against the P450s website (http://p450.antibes.inra.fr/) (Fig. 9).

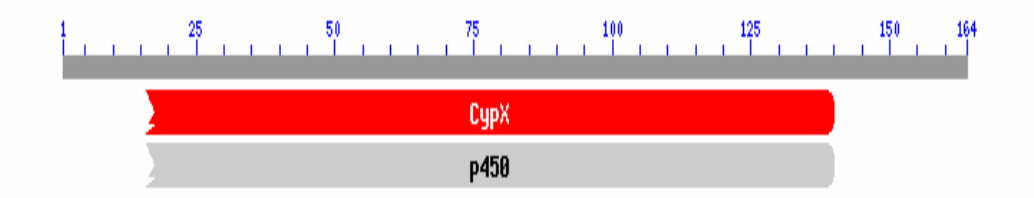


Fig. 9: Conserved putative domain of P450 gene detected after BLAST of *An. funestus* sequenced clone. Red: cluster identification (id) for Cypx, cytochrome P450; Grey: multi-domain conserved domain, P450, cytochrome P450.

The sequences that had no homologies in the database as P450 were excluded from further analysis. The sequence lengths obtained for CYP4, CYP6 and CYP9 after removal of the vector sequence, are shown in Table 1. The sequences from each class were assembled into contigs using Seqman of DNASTAR™ (Lasergene) programme. The contigs were saved as consensus sequences and blasted once more against the P450 site (http://p450.antibes.inra.fr/) to confirm results obtained when the sequences were blasted individually. Table 1 lists 20 *An. funestus* partial CYP4, CYP6 and CYP9 genes that had significant homology to *An. gambiae* P450 entries. A total of 11 CYP4, four CYP6 and five CYP9 partial P450 genes were isolated from *An. funestus* cDNA. This high homology (> 79%) to *An. gambiae* P450s was obtained with all but one (CYP9J14) *An. funestus* P450 sequences. CYP9J14 showed only 55% homology to *An. gambiae* CYP9J5. The sequences were named in accordance with the P450 nomenclature committee (http://drnelson.utmem.edu/CytochromeP450.html) and subsequently deposited into the GenBank for accession numbers (Table 1).

Table 1: Comparison between *An. funestus* and *An. gambiae* CYP4, CYP6 and CYP9 P450 genes. Accession numbers as appears in the GenBank are indicated.

***An. funestus* gene name**	**Closest *An. gambiae* name**	**% amino acid identity to *An. gambiae***	**Accession No.**	**Total cDNA clones**	**Transcript length (coding sequence)**
CYP4C25	CYP4C25	98	AY987353	1	450
CYP4C40	CYP4C35	86	AY648707	4	448
CYP4C41	CYP4C27	93	AY648700	1	450
CYP4D25	CYP4D22	94	AY648701	2	453
CYP4D26	CYP4D15	85	AY648702	2	453
CYP4D27	CYP4D17	86	AY648703	2	435
CYP4G21	CYP4G17	94	AY648704	3	453
CYP4J12	CYP4J10	83	AY648706	6	465
CYP4J11	CYP4J5	79	AY648705	5	459
CYP6AA4	CYP6AA1	84	AY729658	1	375
CYP6M7	CYP6M3	89	AY729659	1	375
CYP6M8	CYP6M2	91	AY729660	1	375
CYP6P9	CYP6P3	86	AY729661	4	375
CYP9J11	CYP9J5	80	AY729662	13	774
CYP9J12	CYP9J3	84	AY729663	7	783
CYP9J13	CYP9J4	84	AY729664	4	744
CYP9J14	CYP9J5	55	AY729665	1	756
CYP9M3	CYP9M2	84	AY729666	8	765

3.3.4 Characterisation of the predicted CYP4 proteins

Deduced amino acid sequence lengths varied from 145 to 155 for CYP4 sequences. The hydrophobic N-terminal region consists of the conserved signature motif FXXGXXXCXG in the heme-binding region (Fig. 10) except for CYP4G21. Amino acid replacements were only found in CYP4G21 within the heme domain where Serine, Threonine and Arginine replaced Isoleucine, Asparagine and Glutamic acid, respectively, in the multiple sequence alignment (Fig. 10). The charge pair consensus (EXXR) within the K-helix is shown in bold.

Another consensus, (aromatic)XXP/D) that is shown in bold in CYP4C27 (Fig. 10) precedes the PERF conserved motif by four residues. The invariant 13 amino acid residues, EVDTFMFEGHDTT that is wholly conserved between positions 1-13 in the multiple sequence alignment is unique to CYP4 family (Bradfield *et al*. 1991).

```
CYP4C40        EVDTFMFEGHDTTTAGMSWALFLLALHPDVQEQVHQEIDSIFN------GSERPATMQDL 54
CYP4C41        EVDTFMFEGHDTTTAGISWVLFLLALHPDVQERVYEEIESIFPT-----GDNRPATMQDL 55
CYP4G21        EVDTFMFEGHDTTAAGSSFVLCLLGIHQHIQDRVYAELRQIFG------DSKRKATFGDT 54
CYP4C25        EVDTFMFEGHDTTSAAISWILLLLGTEPTIQDRIAEEIDQIMGG-----DRERFPTMQEL 55
CYP4J11        EVDTFMFEGHDTTASALVFIFLTLSWEPEIQQRLYQELQQFR---TEKGMADGHFPASDL 57
CYP4J12        EVDTFMFEGHDTTAAAIMFTIVLLATEQDVQERCYKELEELLNGMVTTSSESVRLSVQDY 60
CYP4D26        EVDTFMFEGHDTTTSAISFILLCMAKNPEIQEKVFNEVRNVVGD-----DRKQPVTMAML 55
CYP4D27        EVDTFMFEGHDTTTSAISFLLLSLAKNPDIQQKVFDEVRNIVGD-----DRTRPVTMPML 55
CYP4D25        EVDTFMFEGHDTTTIAISFTLLLLARHPEAQEKVYQEVVDIVGN-----DPYTPLSHRNL 55
               *************: .  : :  :. .   *::   *: ..              .

CYP4C40        NEMKLLERCLKETLRLYPSVSFFGRTLSEDVTLG--GYHVPSGTIIGIHTYNVHRDERFF 112
CYP4C41        NELKLLERCIKEALRLYPSVSFFGRTLSEDIQLG--GYHVPNQTIVGIHAYHVHRDERYY 113
CYP4G21        LEMKYLERVIFETLRMFPPVPMIARKINEDVQLASKNYTIPAGTTVVIGTYKIHRREDLY 114
CYP4C25        NEMKYLEACIKEGLRLYPSVPLIARRLTEDVDID--GYVLPAGTTAMIVVYQLHRNPEVF 113
CYP4J11        NSLKFFDRVIKECLRLWPPVAFISRAVTEEVNLPD-GRTIPKGCIANLHIFDLHRDPAQF 116
CYP4J12        QYLPYLDRVIKESLRLYPPVAFISRATTGEL-VVD-GTIFPHNTITHVHIYDLHRDPAQF 118
CYP4D26        NDMHYLDLVIKETLRXYPSVPMFGRKMLQNTEIN--GKIFPAGSNVVMFPFFLGRDPDYF 113
CYP4D27        NDMHYLDLVIKETLRLYPSVPLIGRKMLQTTDIN--GKTFPAGANLIIMPFFLGRDERYF 113
CYP4D25        QDMKYLEMVIKESLRLYPPVPIIARRFTENVELG--EKIVPEGSNFNIGIMHMHRDPTLF 113
                 :  ::  : * ** :*.*.::.*       :      .*      :    : *    :

CYP4C40        PDAETFDPDRFLPENSERR-HPYAYIPFTAGPRNCIGQ 149
CYP4C41        PEPEKFDPDRFLPENTENR-HPYAYIPFTAGPRNCIGQ 150
CYP4G21        PHPETFNPDNFLPERTQNR-HYYSYIPFTAGPRTASAR 151
CYP4C25        PNPDKFNPDHFLPENCRGR-HPYAYIPFTAGPRNCIGQ 150
CYP4J11        PDPDRFDPDRFLPERVAER-NPFAYVPFTAGPRNCIGQ 153
CYP4J12        PDPERFDPDRFLPEVAEKR-NPYAYVPFTAGPRNCIGQ 155
CYP4D26        PNPEKFDPERFNVETSAEKTNPYQYVPFTAGPRNCIGQ 151
CYP4D27        PDPERFDPERFNVERSAEKTNPYQYIPFYRRP------ 145
CYP4D25        PDPERFDPERFAPDRTMEQSSPYAYVPFSAGPRNCIGQ 151
               *..: *:*:.*  :    :   : *:**   *
```

Fig. 10: Predicted amino acid sequences of *An. funestus* CYP4 P450s. Conserved amino acid residues among P450s are in bold. The heme-binding domain is indicated by an arrow. Amino acid replacements in the heme-binding region of CYP4G21 are boxed. Identical or highly similar residues are shown in asterisks and colons. Missing amino acid residues in within the heme-binding domain in CYP4D27 in the multiple sequence alignment were truncated due to bad sequences.

3.3.5 Characterisation of the predicted CYP6 proteins

Figure 11 shows amino acid sequence alignment of CYP6M7, CYP6M8, CYP6AA4 and CYP6P9. The deduced amino acid sequence lengths for the four sequences were 125. Other conserved amino acid residues are evident in the alignment with other conserved domains characteristic of P450s shown in bold.

```
CYP6M7          LYELALNQDAQDKGRRCVKEVLERHNGELTYDAVMEMHYLDQIPKESLRKYPPVPVHFRT 60
CYP6M8          LYELALNPEVQEQGRKCVREVLKKHNGEMSYDAVVDMKYLDQILNESLRKYPPVPVHFRV 60
CYP6P9          LYELAKNPDIQERLRQEINQAIEENDGQVTYDVAMNIQYLDNVINETLRKYPPVESLSRV 60
CYP6AA4         LYELANNPDIQERVRAEVLEKLKLHDNQITYDALKEMTYLDQVINETLRMYPPVPQLIRV 60
                ***** * : *:: *  : : :: ::.:::**.  :: ***:: :*:** ****    *.

CYP6M7          TSKEYQVPGTKTVLEAGTSVMVPVYAIHRDPEHFPNPDLFDPDRFTPEEEAKRHPYAWTP 120
CYP6M8          ASKDYQVPGTKSVLEAGTAVMVPVHAIHHDPAVFPDPERYDPERFSPEQEAKRHPYAWTP 120
CYP6P9          PSVDYVIPGTKHVIPKRTLVQIPVHAIQHDPEHYPDPERFDPDRFSPEEVKKRHPFTFLP 120
CYP6AA4         ATQPYAVAGTNVTLDRDTMLMVPIYAIHHDATIYPDPKRFDPDRFATDALHSRHTHAFLP 120
                .:  * :.**: .:   * : :*::**::*.  :*:*. :**:**:.:   .**..:: *

CYP6M7          FGEGP 125
CYP6M8          FGEGP 125
CYP6P9          FGEGP 125
CYP6AA4         FGEGP 125
                *****
```

Fig. 11: Predicted amino acid sequences of *An. funestus* CYP6 P450s. Conserved P450 domains are in bold. Identical or highly similar residues are shown in asterisks and colons.

3.3.6 Characterisation of the predicted CYP9 proteins

Deduced amino acid sequence lengths of CYP9 (Fig. 12) varied from 248 to 261 amino acid residues. Gaps created due to the multiple sequence alignment did not contribute to the overall length of the sequences. Five CYP9 clones assembled into two different contigs. Consensus sequences from these contigs were 70% and

55% identical to *An. gambiae* CYP9J5 and were named CYP9J11 and CYP9J14 respectively. The CYP9J14 has also been isolated from the genomic DNA of *An. funestus* (Amenya *et al*., 2005).

```
CYP9J11         FTGSKMRQMFELIVECSANMAKHYREEIRMNGVSSREHEMKDVFTRYANDVIATCAFGIK 60
CYP9J14         ---AKCVTMFQQVVECSSGMVQYYKKQTDTNGQC---YELKEVFSRYTTDVIASCAFGLK 54
CYP9J12         FTGSKMRLMFALIAECGQTMVAHFRSEEQKAGGTGVQLEMKDVMTRFANDVIGTAAFGIK 60
CYP9J13         -------QMFELMSESCQGMVQHLLEEAR-ADESKQVHEMKDIFTRLANDVIASIAFGIQ 52
CYP9M3          FTGSKMRSMFGLLSKSAGDAMDRLVMFSRDKSFT---MELRDLYSRLGNDVMSSISFGVE 57
                       **  : :.              .      *:::: :*  .**:.: :**::

CYP9J11         VDSLKNADNDFYVNGKKMMAFNRPIVMLKLIGFRVVPKLMNWXXLDLFDREQGDYFTEII 120
CYP9J14         VDSFRDSTNGFYTSGQRMIDFARFKVLLKVLAYRLFPWIMEKLEVDLFDRELNSFFAEIV 114
CYP9J12         VDSFRDPTNQFISMARSVTSQESXVKVLKMLGFTFAPKLMXRLNIDFLTPEENRFFXDTI 120
CYP9J13         VNSFSERENDFYKRGKXLLDFTSFWPSIRFMLFMXMPRVMLKLNIELMDKEMCQHFHAMI 112
CYP9M3          VDSLTDRENEFFLKGKRLAQIDG-LPGLKFLMATTIPKVFRFLRLSGMYKDVNEFYLDAV 116
                *:*: :  * *   .: :         ::.:     * ::    :. :  :   .:   :

CYP9J11         RDTLKTREAHGIVRPDMVNLLMQARKGTIKREQRETEDREEVKG--FATVEESDVGQTGK 178
CYP9J14         LETEKTRELQGIVRPDMIHLLMQAKKGILKR-HKEQEE-SLAEG--FATAQESDVGAVDS 170
CYP9J12         LETMRTRADKGIFRPDMIELLMQAKKGSLKHQQAEEPKQEXGTGDGFATVEESHVGRRAH 180
CYP9J13         MDNMKVREEKGIVRNDMINILMQVKRGMLSH-QRDEPD-VKDAG--FATVHESAVGKKAI 168
CYP9M3          SRNIKLRETNRITRPDFIHLLLQARKNTLGAEKHDDET-LQDAG--FSTAQTHAVEQKGE 173
                  . : *  : * * *::.:*:*.::. :   : :        *  *:*..   *

CYP9J11         G--LQMTELEMVAQCLIFFLAGFDTVSTCLTFLAYELTVNKDAQNKLYEEIRATSKSLG- 235
CYP9J14         QHYQTMTQMELVAQCLIFFLAGFDTVANCLTFLAYELTLNRDIQDRLYEEIVATDIDLQ- 229
CYP9J12         D--RVWSDXELIAQAFIFFFAGFETISWTLSFALYELAVNDDIQXRLFEEVHESEQSLEE 238
CYP9J13         T--REWSEKELVAQCFLFFLAGFXTISTALGFLAYELMLHPEVQDRLYEEIVGVDEKLN- 225
CYP9M3          GK-LSWEDIDIAGATASFFFGGIETTTTLLCFASYELSINPSIQDRLRAEIDETREELED 232
                       : :: .    **:.*: * :  * *  *** :: . * :*  *:     .*

CYP9J11         GSSLTYDALQNMQYMDMVVS ETL 258
CYP9J14         QKPLTYEALQHMKYMDMVVS ETL 252
CYP9J12         GKSLSYEKLQSLPYLDMVVS ETL 261
CYP9J13         GKPLNYEAVQGMRYMDMVVS ETL 248
CYP9M3          GKTPTYEILQKMKYLDMVVS ETL 255
                 .. .*: :* : *:********
```

Fig. 12: Predicted amino acid sequences of *An. funestus* CYP9 P450s. Conserved EXXR domain is shown in bold although R is missing. Identical or highly similar residues are shown in asterisks and colons. The missing amino acid residues in CYP9J14 and CYP9J13 were truncated due to bad sequences.

3.3.7 Sequence clustering and phylogenetic analysis

To examine the relationship between *An. gambiae* and *An. funestus* CYP4, CYP6 and CYP9 gene families, multiple sequence alignments of individual P450 families were carried out prior to molecular phylogenetic analysis. *Anopheles gambiae* P450 sequences that were orthologs to the isolated *An. funestus* P450s were retrieved from the P450 website (http://p450.antibes.inra.fr/). Figures 13, 14 and 15 are neighbor-joining trees constructed to illustrate the relationship between *An. funestus* and *An. gambiae* CYP4, 6 and 9 genes. The sequences used in the phylogenetic analysis are of the P450 genes shown in Table 1.

In order to minimise the presence of large gaps created using overall sequence alignment, orthologous genes from *An. gambiae* (Table 1) had their amino acid full-length sequences trimmed and only sequence regions that aligned to the *An. funestus* partial sequences were used for this analysis. The sequences were aligned using ClustalW (BIOEDIT programme (Hall, 1999) and the files converted to MEGA format using text converter (MEGA 2.1, Kumar *et al*., 2001). The gaps within the alignment did not contribute to the overall length of the sequences compared.

Phylogenetic reconstruction using bootstrap values at 1000 generated tree topologies that ascribed the *An. funestus* P450s to orthologous P450s in *An. gambiae*. *Anopheles gambiae* CYP303A1 was included as the outgroup. The consensus phylogenetic tree in Fig. 15 further supports the information generated

by BLAST analysis in which CYP9J14 was only 55% similar to *An. gambiae* CYP9J5. CYP9J14 clustered within the CYP9J subfamily but did not have a corresponding ortholog.

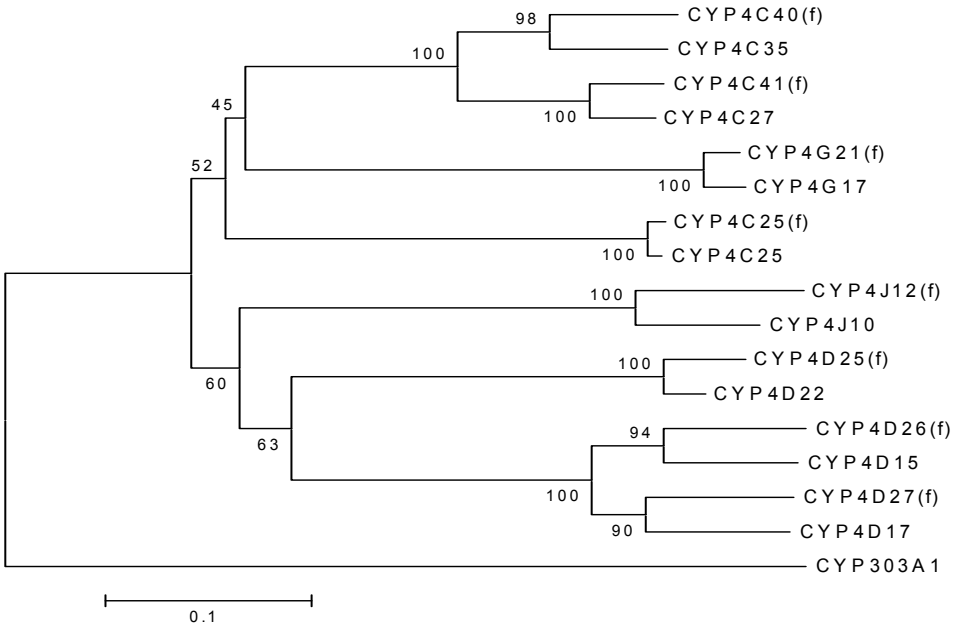


Fig. 13: A neighbor-joining tree showing the relationship between *An. funestus* and *An. gambiae* CYP4 P450s. *Anopheles funestus* sequences are designated as (f).

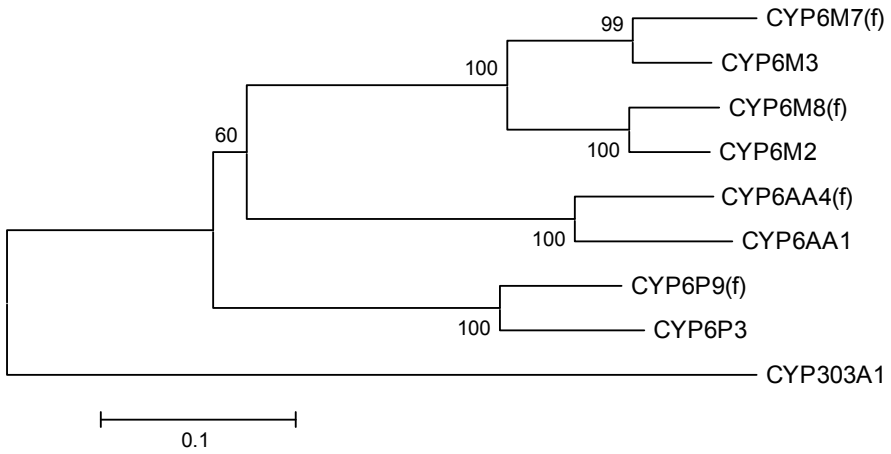


Fig. 14: A neighbor-joining tree showing the relationship between *An. funestus* and *An. gambiae* CYP6 P450s. *Anopheles funestus* sequences are designated as (f).

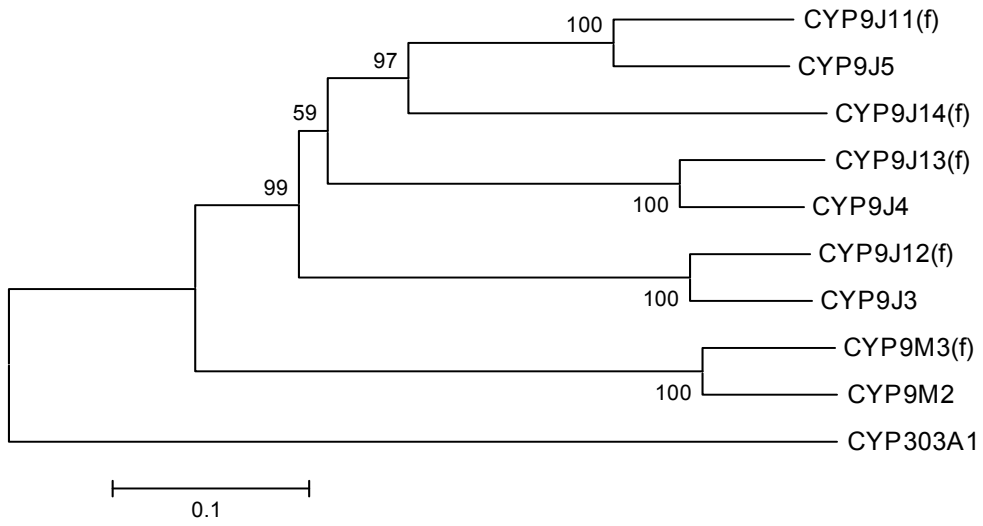


Fig. 15: A neighbor-joining tree showing the relationship between *An. funestus* and *An. gambiae* CYP9 P450s. *Anopheles funestus* sequences are designated as (f).

3.3.8 Intron-exon organization

Genomic DNA sequences were annotated for introns using Mapdraw of DNASTAR™ (Lasergene) programme and blasted against the P450 website (http://p450.antibes.inra.fr/). All intron-exon boundaries of the partial sequences conformed to the canonical GT-AG rule (Mount, 1982). Table 2 shows the splice site of the putative 5' and 3' regions (exon/intron) of the partial *An. funestus* P450 genes. The GT is defined as the 5' donor sequence and AG as the 3' acceptor sequence (Mount, 1982). Table 3 shows a summary of the intron pattern found in *An. funestus* in comparison to *An. gambiae*.

According to the splice site, introns can be divided into three phases. Phase 0 splice site lies between two codons, while phase I site lies one base inside the codon in the 3' direction and phase II intron lies two bases inside a codon in the 3' direction (Tijet *et al*., 2001). CYP4 sequences in *An. gambiae* have been reported to contain all the intron phases described. Within the CYP6 family, all introns were found to be phase I and this is similar to *An. gambiae* where only phase I introns were identified (Ranson *et al*., 2002a). No introns were identified within the CYP9 genomic sequences provided. Subsequently, the sequences were deposited into the GenBank (accession numbers are as presented in Table 3).

Table 2: The splice site of the putative 5' and 3' regions (exon/intron) of partial *An. funestus* P450 sequences.

Gene	5' splice site (exon/intron) **AG/GTRAGT**	3' splice site (intron/exon) **Y_NNYAG/NN**
CYP4J12	GG/**GT**AAGA	TCTCC**AG**/AA
CYP4C40	CG/**GT**ACTG	CCGCT**AG**/AT
CYP4H14	GA/**GT**AAGT	TCACC**AG**/AT
CYP6P1	TG/**GT**AAGA	TCACC**AG**/AA
CYP6P2	TG/**GT**AAGC	ATTTT**AG**/AA
CYP6P9	CG/**GT**ATGA	GTTTC**AG**/AA
CYP6P4	CG/**GT**AAGC	CTTAT**AG**/AA
CYP6P5	CG/**GT**AAAT	ATTGC**AG**/AA
CYP6M1	CG/**GT**ATAA	CTTCC**AG**/AA
CYP6N2	CG/**GT**AAGA	TCGAA**AG**/TA
CYP6Y1	TG/**GT**GGGT	ATTTC**AG**/AA

Table 3: Intron pattern in partial CYP4 and CYP6 gene sequences of *An. funestus*.

P450	**Total No. of introns**	**Intron size in *An. funestus* (bp)**	**Intron size in *An. gambiae* (bp)**	**Intron position**	**Accession number**
CYP4J12	1	74	423	Phase 1	AY648706
CYP4C40	1	100	80	Phase 2	AY648707
CYP4H14	1	71	76	Phase 1	AY987354
CYP6P1	1	69	79	Phase 1	AY987352
CYP6P2	1	73	67	Phase 1	AY987358
CYP6P9	1	57	79	Phase 1	AY729661
CYP6P4	1	68	67	Phase 1	AY987359
CYP6P5	1	81	99	Phase 1	AY987360
CYP6M1	1	64	78	Phase 1	AY987356
CYP6N2	1	75	77	Phase 1	AY987357
CYP6Y1	1	75	76	Phase 1	AY987361

3.4 DISCUSSION

3.4.1 Sequence comparison between *An. funestus* and *An. gambiae* P450s

Due to their key role in metabolism and detoxification processes, the superfamily of cytochrome P450s in insects continues to generate a lot of interest. This is accelerated by the availability of insect genome sequences that have been released or are in the process of being made accessible to the public (Tijet *et al*., 2001; Ranson *et al*., 2002a, b). The importance of cytochrome P450 enzymes in insecticide resistance and particularly the role of CYP4, CYP6 and CYP9 are well documented (Feyereisen *et al.,* 1989; Rose *et al*., 1997; Pridgeon *et al*., 2003; Nikou *et al*., 2003). The current study presents the isolation of partial P450 genes from three classes (CYP4, CYP6 and CYP9) from *An. funestus*. The results showed a high degree of sequence homology among P450s isolated from *An. funestus* with those of *An. gambiae*.

Degenerative PCR was carried out from total RNA isolated from whole mosquitoes of pyrethroid resistant *An. funestus*. This was important to maximise the probability of amplifying mRNA expressed in this strain (Fig. 6). The use of SSCP in screening of clones prior to sequencing was found to be cost-effective compared to the use of restriction enzymes. The various conformational patterns were used to identify and select unique clones for sequencing.

Selection using SSCP worked well for CYP4 and CYP6 clones. However, the CYP9 selection was less successful. This could be due to the fact the technique was not sensitive for the CYP9 amplicon (750 bp). Hayashi, (1991) reported that the sensitivity of PCR-SSCP decreases for DNA fragments longer than 300 bp with a drop of upto 67% for fragments between 300-450bp.

Thirteen cDNAs encoding CYP9J11 were isolated from *An. funestus* after clone selection using SSCP (Table 1). The high frequency of such transcripts might be dependent on the fact that the degenerate CYP9 primer used preferentially amplified this specific gene. It may also be an indication that the CYP9 gene is highly expressed in adult *An. funestus*.

BLAST analysis of *An. funestus* P450 amino acid sequences against those of *An. gambiae* P450s revealed that the two species share high sequence identity and form orthologous groups. Sequence homology by family was based on comparison with the published *An. gambiae* P450 protein sequences (http://p450.antibes.inra.fr/, Ranson *et al*., 2002a; Nikou *et al*., 2003). With the exception of CYP9J14, all *An. funestus* CYP4, CYP6 and CYP9 P450s amino acid sequences showed >75% identity to *An. gambiae* P450s (Table 1). CYP9J14 had no clear ortholog and was only 55% identical to CYP9J5 in *An. gambiae*.

3.4.1.1 Identification of *An. funestus* CYP4 genes

The first P450 isolated in 1958 from rat liver had fatty acid and hydroxylase activity and was designated as CYP4A1 by the P450 nomenclature committee (http://drnelson.utmem.edu/CytochromeP450.html). Incidentally, the first insect P450 isolated from *B. discoidalis* and named CYP4C1, was also assigned to this family based on its I-helix sequence identity to vertebrate CYP4 proteins. However, its overall identity to vertebrate CYP4 proteins was only 32-36% (Bradfield *et al*., 1991). This family of P450s consists of both vertebrate and insect genes (Nelson, 1998). Genes within this family are increasingly being associated with insecticide resistance (reviewed in Feyereisen, 1999; Pridgeon *et al*., 2003).

Currently, there are over 70 subfamilies in CYP4 family (http://drnelson.utmem.edu/CytochromeP450.html). Out of these, *Anopheles* CYP4 genes belong to six subfamilies comprising of C, D, G, H, J and K (Scott *et al*., 1994; Ranson *et al*., 2002a). Other subfamilies comprise of genes encoding CYP4 family in insects such as *M. domestica*, *B. germanica*, *B. discoidalis* and *D. melanogaster* (Bradfield *et al*., 1991; Dunkov *et al*., 1996; Tijet *et al*., 2001; Wen *et al.,* 2001). A BLASTX search of *An. funestus* clones obtained after PCR with degenerate CYP4 primers identified 12 CYP4 genes consisting of C, D, G and J subfamilies (Amenya *et al*., 2005). These genes share optimal translated protein homology as shown in Fig.10. The number of CYP4 P450 genes identified in *An. funestus* is 41% of the total number found in *An. gambiae* (29 genes) (Ranson *et*

al., 2002a, b). The method used to isolate CYP4 genes could therefore be considered successful.

Multiple sequence alignment of the inferred amino acids of the 10 CYP4 gene fragments, obtained from cDNA, indicated a high degree of similarity among the sequences when considering the signature motifs found in CYP4 family (Fig. 10). It is not clear at this stage whether the replacement of three amino acids within the heme-binding domain in CYP4G21 (Fig. 10) may affect its function. This is can only be confirmed through characterisation of its protein. However, studies have reported that the reduced affinity for carbon monoxide in CYP74 in *Arabidopsis* is due to the missing residues involved in oxygen-binding and electron transfer (Werck-Reichhart, *et al.,* 2002).

Of all the CYPs in insects, the CYP4C25 gene has been identified as highly conserved by the P450 nomenclature committee (D. Nelson, personal communication). CYP4C25 is currently only found in *An. gambiae* and *An. funestus*. The *An. funestus* CYP4C25 is 98% identical to the CYP4C25 found *An. gambiae* with only two amino acids difference. This conservation could be due to their role in metabolising endogenous compounds (Amenya *et al*., 2005).

3.4.1.2 Identification of CYP6 genes in *An. funestus*

Four CYP6 genes were isolated from *An. funestus* cDNA (Table 1). The length of deduced amino acid sequence of these genes was 125 (Fig. 11). This length is approximately a quarter of the total length of *An. gambiae* CYP6 proteins (Ranson

et al., 2002a). No allelic variations have so far been reported within this family of P450s in *Anopheles* although they have been reported in CYP6D1 of *M. domestica* (Tomita and Scott, 1995; Ranson *et al*., 2002a).

A total of 22 P450s in *D. melanogaster* belong to the CYP6 family (Adams, *et al*., 2000; Tijet *et al*., 2001) while 28 genes have been identified in *An. gambiae* through PCR and annotation of the genome (Ranson *et al*., 2002a). Twelve partial CYP6 genes have been isolated from both the genomic and cDNA templates in *An. funestus* (Amenya *et al*., 2005). This is 42% of what has been isolated in *An. gambiae*. A number of insecticide resistant insects have showed over-expression of gene(s) within this family (Liu and Scott, 1998; Nikou *et al.*, 2003; Rodpradit *et al*., 2005).

Although Ranson *et al*., (2002a) reported seven subfamilies within CYP6 family as being unique to *An. gambiae*, the present study has shown that these subfamilies are also present in *An. funestus* as a number of CYP6 genes isolated so far are orthologs of *An. gambiae*.

3.4.1.3 Identification of CYP9 genes in *An. funestus*

Using cDNA from *An. funestus*, five CYP9 genes were isolated. Three CYP9 genes have also been isolated from *An. funestus* genomic bringing the total number of CYP9 genes isolated in *An. funestus* to 7 (Amenya *et al*., 2005). Eight CYP9 were recorded in *An. gambiae* (http://p450.antibes.inra.fr/). Therefore, it

can be assumed that the majority of CYP9 genes have been isolated in *An. funestus*. In insects species the CYP4 and CYP6 families are bigger than the CYP9 family (Ranson *et al*., 2002a). Other than the involvement of CYP9 genes in insecticide resistance which is well documented, little is known about its other functions in insects (Pittendrigh *et al*., 1997; Stevens *et al*., 2000).

The CYP9J14 sequence is considered novel in *An. funestus* for the purpose of classification within the CYP9J subfamily. This is based on the current naming criteria of P450s that a subfamily shares at least 55% homology (Nelson *et al*., 1996). CYP9J14 may represent recent gene duplication from *An. gambiae* CYP9J5. Therefore, sequencing of full-length gene might be an appropriate approach for a better understanding of gene duplication processes in these two species. This will also confirm its relationship with other CYP9 genes.

3.4.1.4 Intron-exon organisation

The correlation between intron size and position conservation between *An. funestus* and *An. gambiae* P450s was made possible during the analysis. In all the genes, the canonical GT-AG rule was obeyed. This was also similar to orthologous gene in *An. gambiae* (http://p450.antibes.inra.fr/). The fact that no introns were identified in CYP9 genomic sequences suggests that the partial sequences currently available for *An. funestus* are not within the intron regions. The conservation of intron position might indicate their importance in

transcription regulation events and the conservation of gene structure between species.

3.4.2 Phylogenetic comparison between *An. funestus* and *An. gambiae* P450s

Historically, *An. gambiae* and *An. funestus* are grouped under the same subgenus, *Cellia* but belong to Pyretophorus and Myzomyia series respectively (Gillies and De Meillon, 1968). The morphological and cytogenetic divergence places *An. gambiae* and *An. funestus* in two different clades (Green, 1982; Sharakhov *et al*., 2002; Harbach, 2004). These two species groups are morphologically distinguishable from each other in all their life stages and can be identified within their species groups by PCR (Scott *et al*., 1993; Koekemoer *et al*., 2002). Chromosomal inversion studies between the two species groups revealed that since divergence from a common ancestor approximately five million years ago, there have been changes in gene order with corresponding changes in the length of autosomes as well as nucleotide substitutions depending on the structure and function of particular proteins (Sharakhov *et al*., 2002).

The current study has provided further insight into the relationship between *An. funestus* and *An. gambiae* P450 genes using molecular phylogenetic analysis. The phylogenetic trees generated with partial CYP4, CYP6 and CYP9 sequences support evidence of a close relationship of P450 genes between these two species. While housekeeping genes are known to be constitutively expressed in organisms and involved in the basic functions needed in a cell, insecticide resistant genes are

believed to arise from random variation or by insecticide selection and increased mutation rates (Devonshire and Field, 1991; Danielson *et al*., 1997). The diversity of cytochrome P450 superfamilies is currently well established in a number of insects and is believed to evolve through gene duplication, gene conversion, gene loss and lateral transfer processes (reviewed in Nelson, 1999; Tijet *et al.*, 2001; Ranson *et al*., 2002a).

The CYP4G21(f) and CYP4G17 clustered with the CYP4C subfamily of *An. funestus* and *An. gambiae*, respectively. The clustering between the two subfamilies has been shown with different insect species (Dunkov *et al.,* 1996; Tarès *et al*., 2000). In most of the insecticide resistant strains of insects, the genes reported to be over-expressed in CYP4 family belongs to either of these two subfamilies (Pittendrigh *et al*., 1997; Pridgeon *et al*., 2003). The clustering of the two subfamilies in the phylogenetic tree can be explained by the theory of gene duplication, which leads to diversity of P450 genes thus providing an opportunity for divergence in substrate specificity (Nelson and Strobel, 1987). Similarly, their role in insecticide resistance could be as a result of duplication events involving the entire promoter or enhancer region of the two genes resulting in a common regulation (Hung *et al*., 1996). An example of this event was found in CYP6B4 and CYP6B5 genes in *Papilio glaucus*. These genes clearly represent gene duplication resulting in paralog genes yet both induce furanocoumarin (Hung *et al*., 1996). Therefore, the CYP4G and CYP4C subfamilies could also be assumed to maintain their regulatory attributes after duplication.

Phylogenetic analysis of CYP4, CYP6 and CYP9 P450 genes between *An. funestus* and *An. gambiae* revealed high similarity. Bootstrap values were above 93 with the majority at 100. The high values obtained from the bootstraps of orthologous genes showed that the divergence of these genes in the two species was slow. Considering the conserved intron organisation and the high level of amino acid identity in the CYP6 genes in these two species of *Anopheles*, it is possible that these genes originated from a common P450 gene through several gene duplications. This could also explain the clustering of CYP6 genes on chromosome 3R and 2R in *An. gambiae* and *D. melanogaster,* respectively (Tijet *et al*., 2001; Ranson *et al*., 2002a). The tree illustration on the relationship between *An. funestus* and *An. gambiae* CYP9J subfamily supported the BLAST results of the CYP9J14 in which the gene did not have a corresponding ortholog. The CYP9J14 has relatively lower nucleotide and amino acid sequence identity than the two other othologs, CYP9J5 and CYP9J11 in *An. gambiae* and *An. funestus,* respectively.

It has been postulated that P450 genes with near identical sequence similarity and clustering phylogenies have similar metabolic profiles (Williams *et al*., 2004). Consequently, it could also be assumed at this stage that P450 genes in *An. funestus* and *An. gambiae* a have similar substrate specificity and function. This kind of investigation will only be possible once full-length sequences of *An. funestus* P450s are available.

CHAPTER 4

EXPRESSION OF P450 GENES IN A PYRETHROID RESISTANT STRAIN OF ANOPHELES FUNESTUS

4.1 INTRODUCTION

As detailed in Chapter 1, metabolism of insecticides by P450 enzymes is a major mechanism in pyrethroid resistance. This is due to the over-expression of one or more P450 genes. It is assumed that when expression is higher in a pyrethoid resistant compared with a susceptible strain, the P450 gene involved is resistance related (Bergé *et al*., 1998). However, the activity of the over-expressed P450 must be determined because its over-expression does not necessarily prove that it is the enzyme responsible insecticide metabolism (reviewed in Scott, 1999). This chapter reports on the identification of an over-expressed P450 gene (CYP6P9) in the pyrethroid resistant strain of *An. funestus* in comparison to the susceptible FANG strain. These findings constitute a major step forward in the understanding of the molecular basis by which *An. funestus* becomes resistant to pyrethroid insecticides. In the long-term, the information will be useful in the designing of effective counter-measures for resistance by vector control programs.

4.2 MATERIALS AND METHODS

Partial P450 genes isolated in Chapter 3 were used as probes in the expression studies. Also included were plasmids isolated from *An. funestus* genomic DNA (Amenya *et al*., 2005) and the CYP6Z1 plasmid (accession number AF487535) that was found to be over-expressed in *An. gambiae*. Dr. Ranson, Liverpool School of Tropical Medicine, supplied these plasmids. Total RNA was isolated from FUMOZ-R and FANG for expression analysis as outlined in Chapter 2. Serially diluted RNA was spotted onto nylon membranes and probed using either cocktail probes from CYP4, CYP6 or CYP9 genes to identify the class of P450 that was over-expressed. To identify the over-expressed gene, individual genes making up the cocktail were used to probe RNA on dot blots. Northern blot was carried out using CYP6P9 probe to validate the results obtained by dot blots. Equal amount of RNA loading was confirmed using a housekeeping gene, ribosomal surface protein 7 (rsp7) in both dot blots and northern blot analyses.

Semi-quantitative PCR was carried out to determine expression levels of CYP6P9 gene in the various developmental stages of *An. funestus* and to compare sex expression in the FUMOZ-R and FANG strains. Quantitative real time PCR was used to validate the results obtained with blot analysis and semi-quantitative PCR.

4.3 RESULTS

4.3.1 Expression of CYP6 gene in FUMOZ-R and FANG

Initial expression studies were carried out using dot blot analysis. Hybridisation was carried out independently with either the cocktail probes of CYP4, CYP6 or CYP9 genes. The CYP4 cocktail probes consisted of 12 genes (CYP4C25, CYP4C41, CYP4C40, CYP4C36, CYP4D26, CYP4D27, CYP4D25, CYP4G21, CYP4H14, CYP4J11, CYP4J9 and CYP4J12). The CYP6 cocktail probes consisted of 6 genes (CYP6P1, CYP6P2, CYP6P9, CYP6Z1, CYP6M8 and CYP6M7) while the CYP9 cocktail contained 5 genes (CYP9J12, CYP9J13, CYP9J11, CYP9L2/L3 and CYP9M3). The RNA (25 μg) from both FUMOZ-R and FANG was serially diluted to a final concentration of 0.049 μg. The diluted RNA were spotted onto nylon membrane. Visual signals were only obtained from the blots probed with CYP6 cocktail probes (Figure 16A). CYP4 and CY9 probes gave no visual signal (data not shown). A strong signal was observed in FUMOZ-R at 25 μg concentration of RNA with undetectable signal in FANG indicating that the expression of CYP6 class is significantly higher in FUMOZ-R. The rsp7 probe was used to normalise the amount of FUMOZ-R and FANG RNA loaded (Fig. 16B). There was equal intensity in both the blots of FUMOZ-R and FANG RNA probed with rsp7.

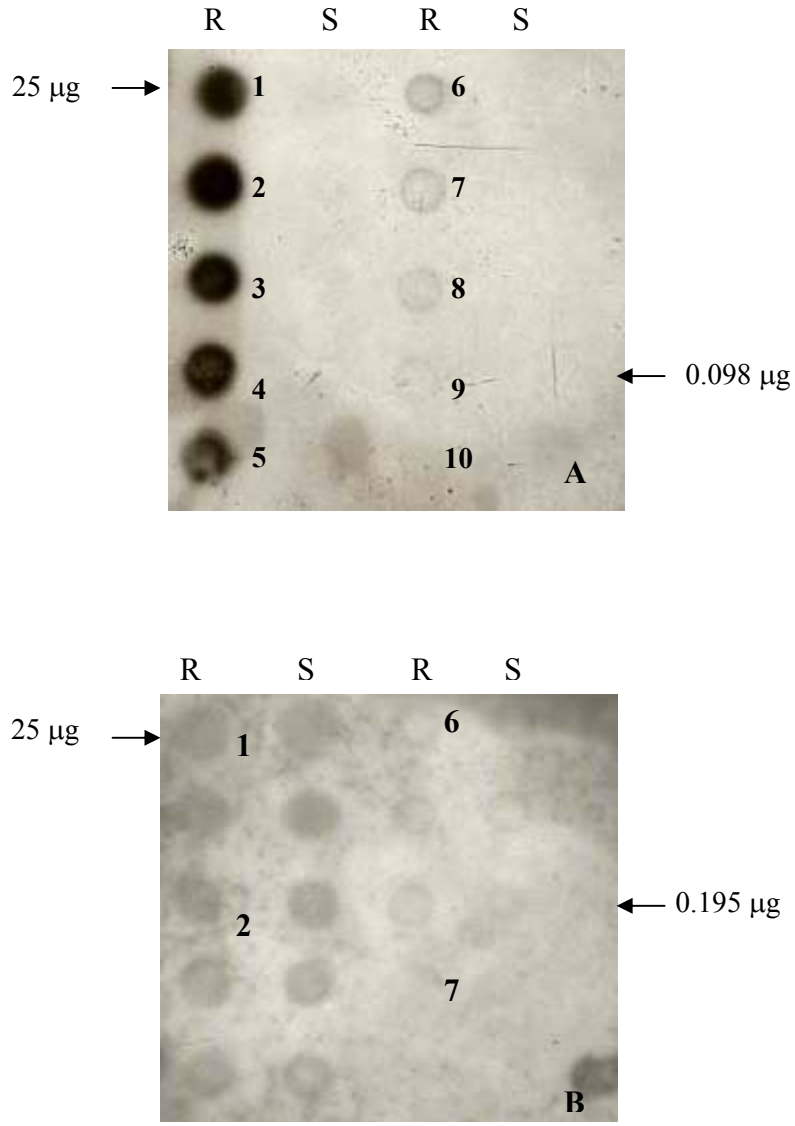


Fig. 16: Dot blot analysis of 3-day old adult FUMOZ-R (R) and FANG (S) RNAs. (A) Autoradiogram after 5 hours exposure, showing hybridisation results with cocktail CYP6 probes. (B) Autoradiogram after 5 hours exposure, showing hybridisation result with rsp7 probe. RNA concentrations (1 = 25 µg, 2 = 12.5 µg, 3 = 6.25 µg,4 = 3.125 µg, 5 = 1.56 µg, 6 = 0.78 µg, 7 = 0.39 µg, 8= 0.195 µg, 9 = 0.098 µg, 10 = 0.049 µg).

4.3.2 A CYP6P9 gene is over-expressed in FUMOZ-R

Independent hybridisations were carried out using individual genes that made up the CYP6 cocktail probes. The probes were used to identify the over-expressed gene and compare its expression levels between FUMOZ-R and FANG. The RNA starting concentration was doubled (50 μg) to provide a visual signal for the lower-expressed Fang sample and was serially diluted to a final concentration of 0.098 μg and spotted on the membrane. Results revealed a strong signal with the CYP6P9 gene in FUMOZ-R with detectable levels in FANG RNA (Fig. 17A). Signals were not observed on the blots that were probed with CYP6P1, CYP6P2, CYP6Z1, CYP6M8 and CYP6M7 genes (data not shown). The expression fold was calculated by visually comparing the signal intensity of the blots in FUMOZ-R to that of FANG. The intensity of the first blot in FANG (50 μg) was similar to the tenth blot in FUMOZ-R (0.098 μg) after serial dilution. The CYP6P9 gene was therefore expressed at approximately 500-fold higher levels in the FUMOZ-R strain compared with the FANG strain. The variation in the expression levels of the CYP6P9 gene was normalised using rsp7 gene (Fig. 17B). Variation observed between spots 4 and 5 (Resistant versus Susceptible) is discussed further in section 4.4.1.1.

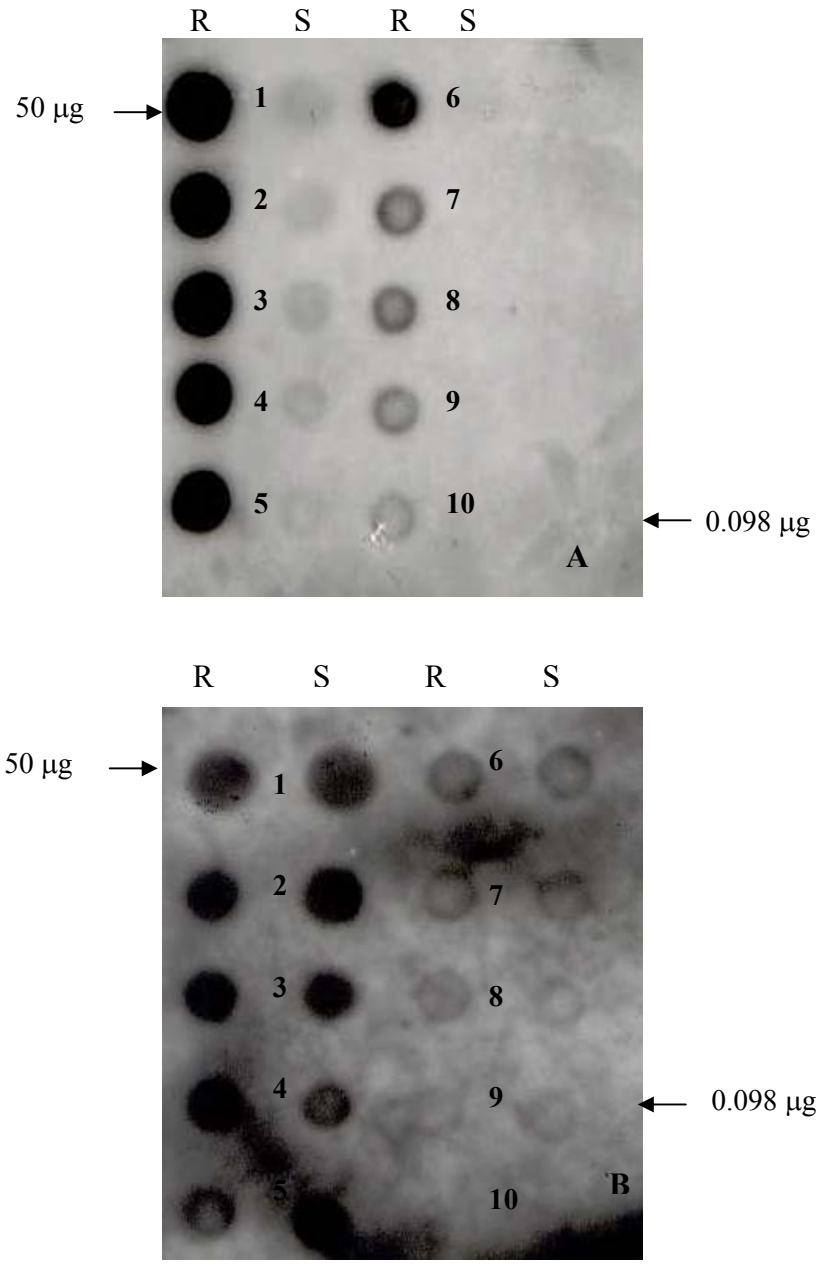


Fig. 17: Dot blot analysis of 3-day old FUMOZ-R (R) and FANG (S) RNAs.

(A) Autoradiogram after overnight exposure, showing hybridisation results with CYP6P9 probe. (B) Autoradiogram after overnight exposure, showing hybridisation result with rsp7 probe. RNA concentrations (1 = 50 µg, 2 = 25 µg, 3 = 12.5 µg, 4 = 6.25 µg, 5 = 3.125 µg, 6 = 1.56 µg, 7 = 0.78 µg, 8 = 0.39 µg, 9 = 0.195 µg, 10 = 0.098 µg).

To confirm the expression of CYP6P9 gene, northern blot analysis was carried out using 5 μg of RNA from both FUMOZ-R and FANG. From the results, the CYP6P9 hybridised to an abundant transcript in FUMOZ-R when compared to FANG (Fig.18A). CYP6P9 expression in the FUMOZ-R strain gave much higher signal intensity on the membrane than the FANG strain (Fig.18A). The same filter was stripped and hybridised to rsp7 probe to normalise the amount of RNA loaded for both strains (Fig. 18B).

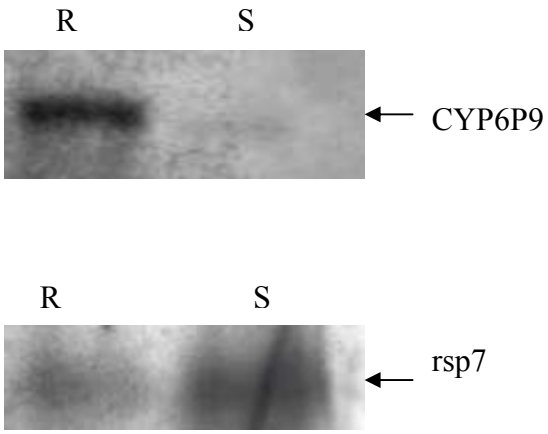


Fig 18: Northern blot analysis of CYP6P9 mRNA levels in FUMOZ-R (R) and FANG (S) strains. RNA (5 μg) were loaded in each lane (A) CYP6P9 probe (B) rsp7 probe.

4.3.3 Expression of CYP6P9 gene in four life stages

Semi-quantitative RT-PCR was used to evaluate the expression profile of the CYP6P9 gene. This was carried out in eggs, 3rd instar larva, pupa and 3-day old males and females from both FUMOZ-R and FANG cDNA using CYP6P9 gene specific primers. Two primer pairs were designed and tested and the PCR

amplification products corresponding to 170 bp and 103 bp were obtained using the RTF1-F/R and RTF2-F/R primer pairs respectively. The expression level of CYP6P9 gene in the early life stages was compared between the two strains.

The results showed varying expression of CYP6P9 gene in the life stages of FUMOZ-R and FANG strains using both primers (Fig. 19). The first primer pair (RTF1) showed no amplified fragments when using eggs as the biological material. Amplification using the rsp7 primers amplified the appropriate fragment in both the resistant and susceptible strains confirming the presence of cDNA. Fragments amplified when using larvae and pupae as biological material were visually brighter in the resistant strain when compared to the susceptible strain (Fig. 19). The same pattern was observed when using adult stages. No amplification was observed in the susceptible strain, while the resistant strain showed amplified fragment (Fig. 20). Amplification of the rsp7 fragment in the susceptible strain indicated that lack of amplification was not due to the absence of template.

Comparison within the resistant strain using this primer set showed an increase in fragment intensity in the larvae when compared to pupae (Fig. 19). The resistant males also showed more intense fragments when compared to females (Fig. 20).

The second primer set (RTF2) showed similar results when comparing resistant and susceptible life stages. As with the first primer pair, there was no amplification when using cDNA extracted from FANG eggs (Fig. 19). The

resistant (FUMOZ-R) eggs in contrast showed a very faint fragment. Again, the larva and pupa from the resistant strain had higher intensity than the susceptible material. Similar results were obtained when comparing adult stages (Fig. 20). This primer pair was able to amplify a CYP6P9 fragment in the susceptible strain even though it was extremely faint. However, comparisons within the resistant strain using these primer pairs showed opposite results compared to the first primer set. Visually the pupa-amplified fragment was more intense than the larva and no difference was observed between the adult males and females.

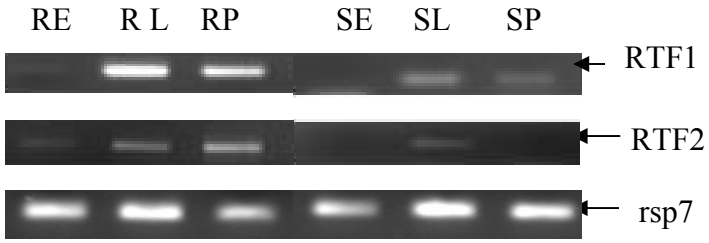


Fig. 19: Semi-quantitative expression of CYP6P9 and rsp7 mRNAs in FUMOZ-R and FANG life stages. The RT-PCR was carried out using RTF1, RTF2 and rsp7 primers. RE (FUMOZ-R eggs); RL (FUMOZ-R larva); RP (FUMOZ-R pupa); SE (FANG eggs); SL (FANG larva); SP (FANG pupa).

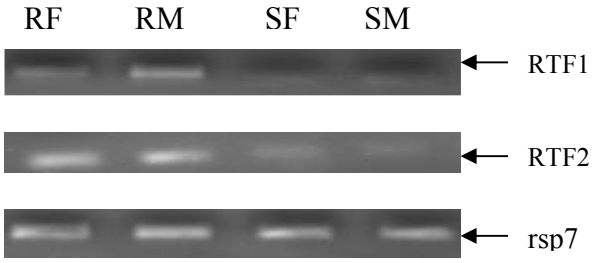


Fig. 20: Semi- quantitative expression of CYP6P9 and rsp7 mRNAs in (FUMOZ-R) and (FANG) adults. The RT-PCR was carried out using RTF1, RTF2 and rsp7 primers. RF (FUMOZ-R females); RM (FUMOZ-R males); SF (FANG females); SM (FANG males).

4.3.4 Verification of transcript levels by quantitative real time PCR

High CYP6P9 mRNA levels are associated with pyrethroid resistant *An. funestus*. This was revealed by the high level of over-expression of this gene in FUMOZ-R compared to FANG using dot blot, northern blot and semi-quantitative analysis. Independent verification of the transcript level visualised in the three methods was carried out using qRT-PCR. The results reported in this section were obtained using the SYBR Green chemistry.

The single peaks on the melt curves were generated using RTF1 (Fig. 21) and qrsp7B (Fig. 22) primer pairs confirmed the specificity of amplification products using the plasmids and cDNAs as templates.

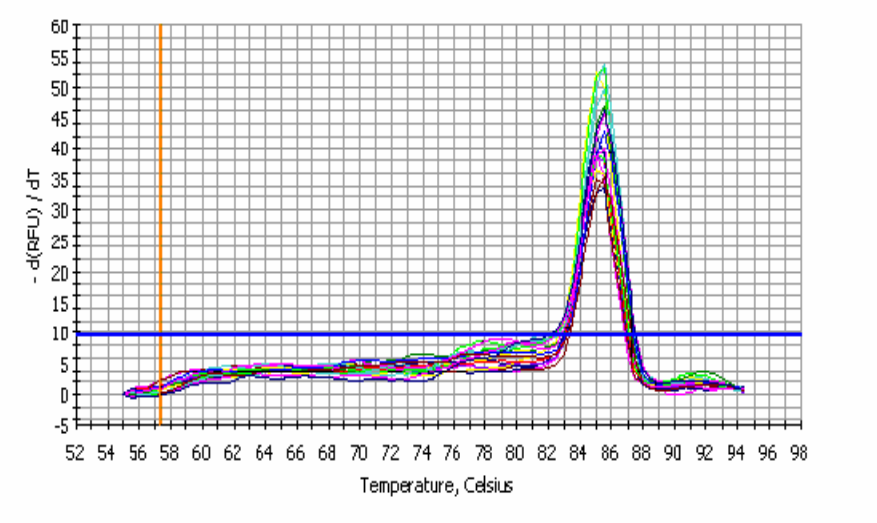


Fig. 21: Melt curve for CYP6P9 standard plasmid. Melting temperature of the primer is 85 ^{o}C.

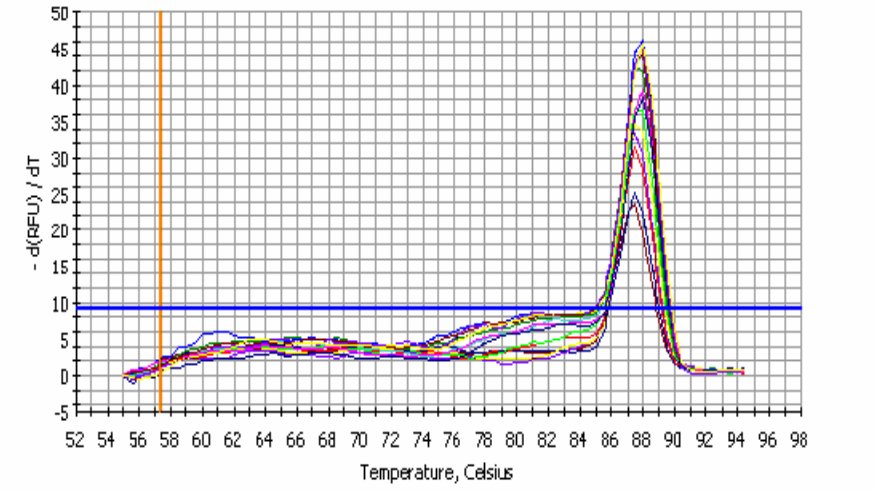


Fig. 22: Melt curve for rsp7 standard plasmid. Melting temperature of the primer is between 87.5 ^{o}C –88 ^{o}C.

Target copy number for the CYP6P9 and rsp7 genes were calculated as described in Chapter 2. The initial target copy number for CYP6P9 gene was calculated as 267.8 copies/fg while that of rsp7 gene was 270.4 copies/fg. Standard curves for

the CYP6P9 and rsp7 plasmids were generated based on the linear relationships between the log concentration (as copy numbers) and their corresponding threshold cycles (C_T) (Figs. 23 and 24). Fluorescence acquisition by PCR of the plasmids is in Appendix C.

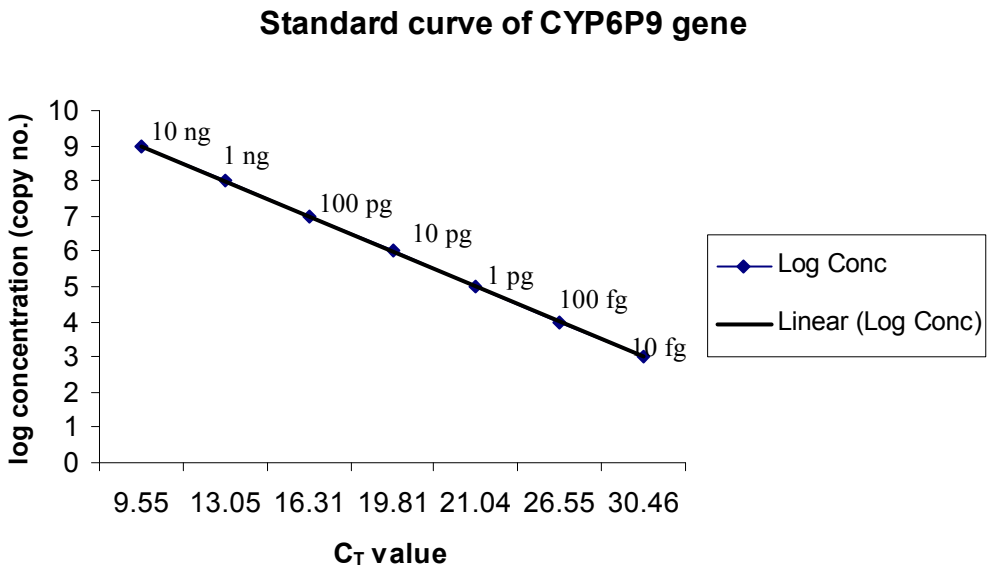


Fig. 23: The relative standard curve obtained with the threshold cycle (C_T) values of CYP6P9 plasmid versus the log of each initial concentration.

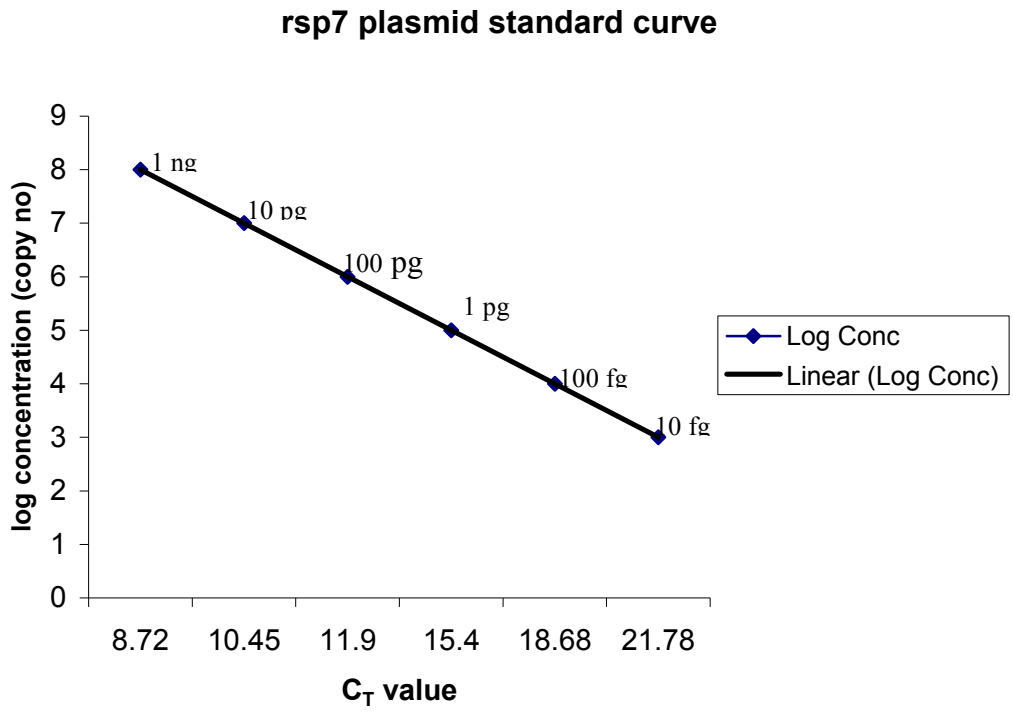


Fig. 24: The relative standard curve obtained with the threshold cycle (C_T) values of rsp7 plasmid versus the log of each initial concentration.

The copy numbers in each of the cDNA samples were interpolated from the standard curves of the respective genes based on the C_T values. Copy numbers were calculated using the equations obtained from the standard curves: For CYP6P9 gene, y = -3.396x + 41.613 and the rsp7 gene, y = - 2.617x + 30.327, where 41.613 and 30.327 represents the intercepts of the relative standard curves of CYP6P9 and rsp7 respectively. The slopes of standard curves for CYP6P9 and rsp7 genes were -3.396 and - 2.617 respectively. The correlation coefficients for the CYP6P9 and rsp7 standard curves were 0.992 and 0.989 respectively. Transcript copy numbers for each cDNA sample were normalised with the respective rsp7 copy number, the mean copy number and the standard deviations calculated. Table 4 shows the normalised copy number, mean copy number,

experimental replicates and standard deviations. The mean transcript copy numbers were used to draw graphs.

Table 4: Table showing normalised copy number, mean copy number, biological replicates and standard deviations. FUMOZ-RM (3-day old FUMOZ-R males); FUMOZ-RF (3-day old FUMOZ-R females); FUMOZ-RL (3rd instar FUMOZ-R larva); FANG-L (3rd instar FANG larva); FUMOZ-R14DM (FUMOZ-R 14-day old males); FUMOZ-RF14D (FUMOZ-R 14-day-old females).

CDNA sample source	Replicates	Normalised copy number	Mean copy number	Standard deviation
FUMOZ-RM	2	118.44 46.43	82.435	7.0
FUMOZ-RF	2	23.389 13.49	18.4395	50.9
FUMOZ-RL	2	6.37 11.41	8.89	3.56
FANG-L	2	1.135 0.51	0.823	0.44
FUMOZ-R14DM	2	27.14 10.5205	18.83	11.75
FUMOZ-R14DF	2	6.018 4.479	5.248	1.09

To ensure that the rsp7gene was indeed expressed equally in *An. funestus* regardless of the strains used, a comparison was done on copy number and C_T values between the resistant and susceptible strains. Table 5 clearly shows that the copy number between the strains are similar and the C_T values in Fig. 25 are

similar as reported in other publications (Nikou *et al*., 2003). After this confirmation, analysis was continued.

Table 5: Table showing C_T values for FUMOZ-R and FANG rsp7 gene.

Sample	**Concentration (ng)**	**C_T value**
FUMOZ-R 1	100	21.02
FUMOZ-R 2	100	21.12
FUMOZ-R 3	100	20.88
FANG 1	100	20.71
FANG 2	100	21.02
FANG 3	100	20.98

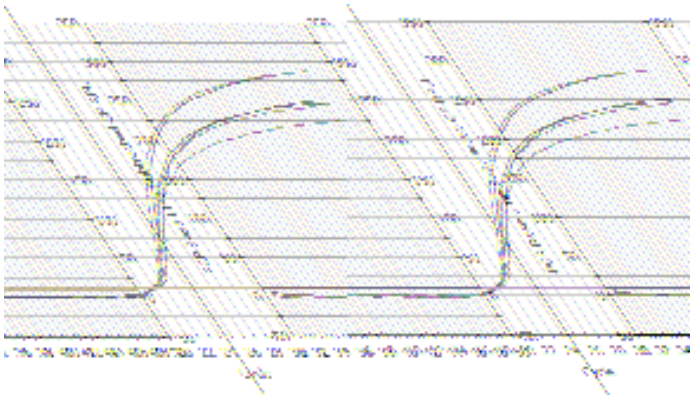


Fig. 25: Fluorescence acquisition graph showing C_T cycles for FUMOZ-R and FANG rsp7 gene. Red line: PCR base line subtracted curve fit. The curve was generated based on the C_T cycles represented on Table 5.

Quantitative RT-PCR data was analysed using two-sample t-test. Expression of CYP6P9 was 4.5-fold higher in 3-day old FUMOZ-R males than females of the same age (Fig. 26) and 3.5-fold higher in the 14-day old males than 14-day old

females (Fig. 27). Statistical analysis at 95 % confidence interval showed that the 3-day old FUMOZ-R male to females had a p = 0.2203 and 14-day old-FUMOZ-R males to females (p = 0.2452). However, the differences were not statistically significant as p values were greater than 0.05. In the larva, CYP6P9 gene was 10.8-fold higher in FUMOZ-R compared to FANG (Fig. 26). The difference was not statistically significant, as the p value was 0.0864. A decrease in expression was observed between 3-day old and 14-day old males and females of FUMOZ-R. Expression was 4.4 fold higher in 3-day old males compared to 14-day males and 3.5 fold higher in 3-day old females compared to 14-day old females. However, this decrease was not statistically significant in the males at 3-day old and 14-day old (p = 0.5366) and the females at 3-day old and 14-day old (p = 0.1100). In all the tests carried out, the calculated t value was lower than the critical t value.

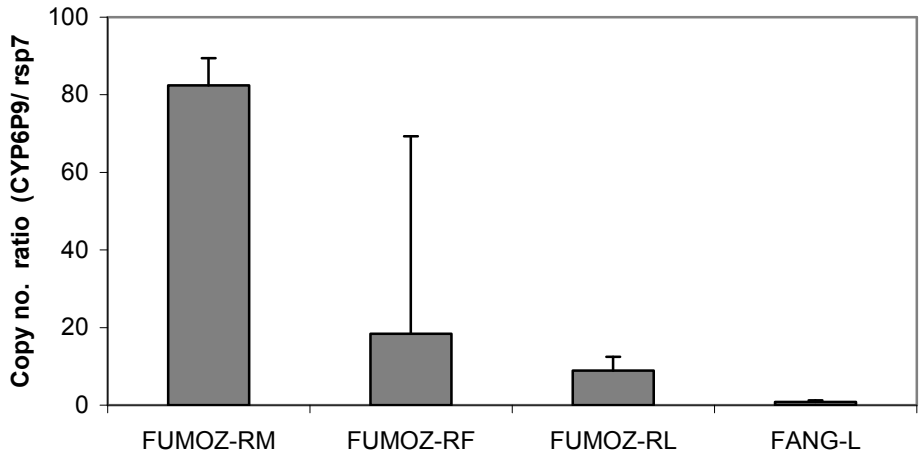


Fig. 26: Expression of CYP6P9 gene using SYBR Green qRT-PCR.

Error bars = standard deviation. FUMOZ-RM (3-day old FUMOZ-R males); FUMOZ-RF (3-day old FUMOZ-R females); FUMOZ-RL (3rd instar FUMOZ-R larva); FANG-L (3rd instar FANG larva).

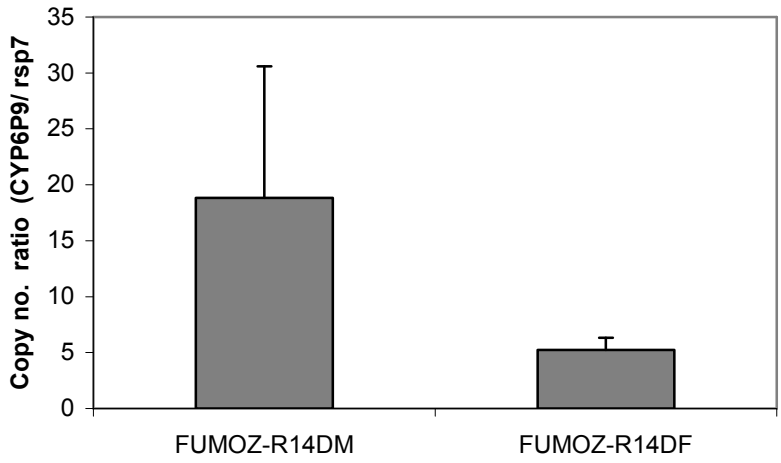


Fig. 27: Expression of CYP6P9 gene in 14-day old FUMOZ-R males and females using SYBR Green qRT-PCR. Error bars represent the standard deviation. FUMOZ-R14DM (FUMOZ-R 14-day old males); FUMOZ-RF14D (FUMOZ-R 14-day old females).

It was not possible to estimate the CYP6P9 copy numbers in FANG males and females due to the presence of primer-dimers on the melt curves (Fig. 28).

Amplified PCR products of FANG males and females showed faint bands on agarose gel electrophoresis (Fig. 29). Optimisation of the PCR conditions was without success.

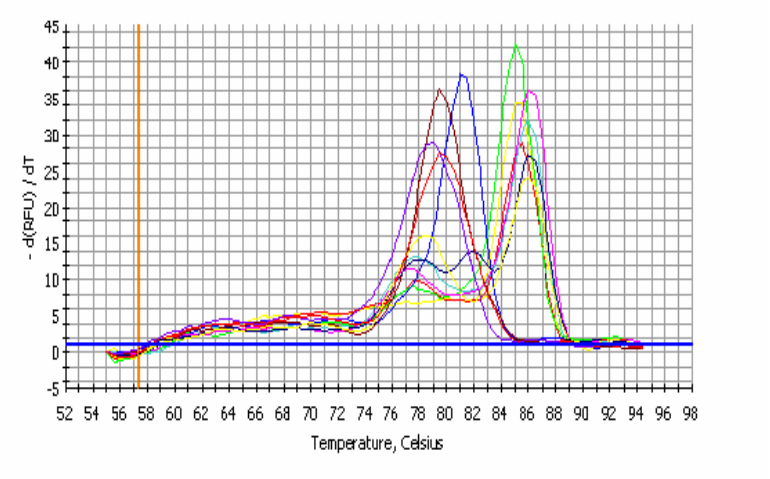


Fig. 28: Melt curves showing primer dimers for FANG males and females.

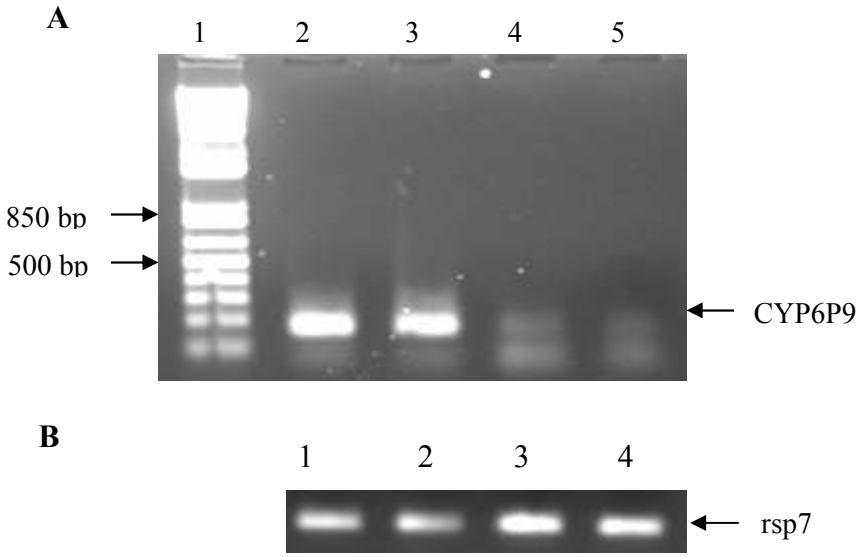


Fig. 29: Expression of CYP6P9 and rsp7 genes in males and females of FUMOZ-R and FANG. PCR products of the real time assay described in Fig. 26 were analysed on a 1% (w/v) agarose gel and stained using EtBr. The presence of primer dimers was noted in the FANG samples. (A) CYP6P9 gene Lane1: 1 kb plus DNA size marker (Invitrogen), Lane 2: RF; Lane 3: RM Lane 4: SF; Lane 5: SM. (B) rsp7 gene. Lane 1: RF; Lane 2: RM Lane 3: SF; Lane 4: SM.

Although semi-quantitative PCR resulted in a single band of 170 bp in the pupa of both FUMOZ-R and FANG (Fig. 19), quantitative real time PCR revealed two bands of 300 bp and the expected 170 bp while in FANG, a band of 300 bp was amplified (Fig. 30). As a result, the pupal results were not analysed.

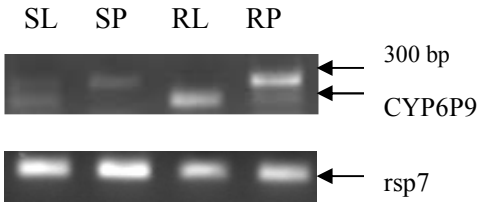
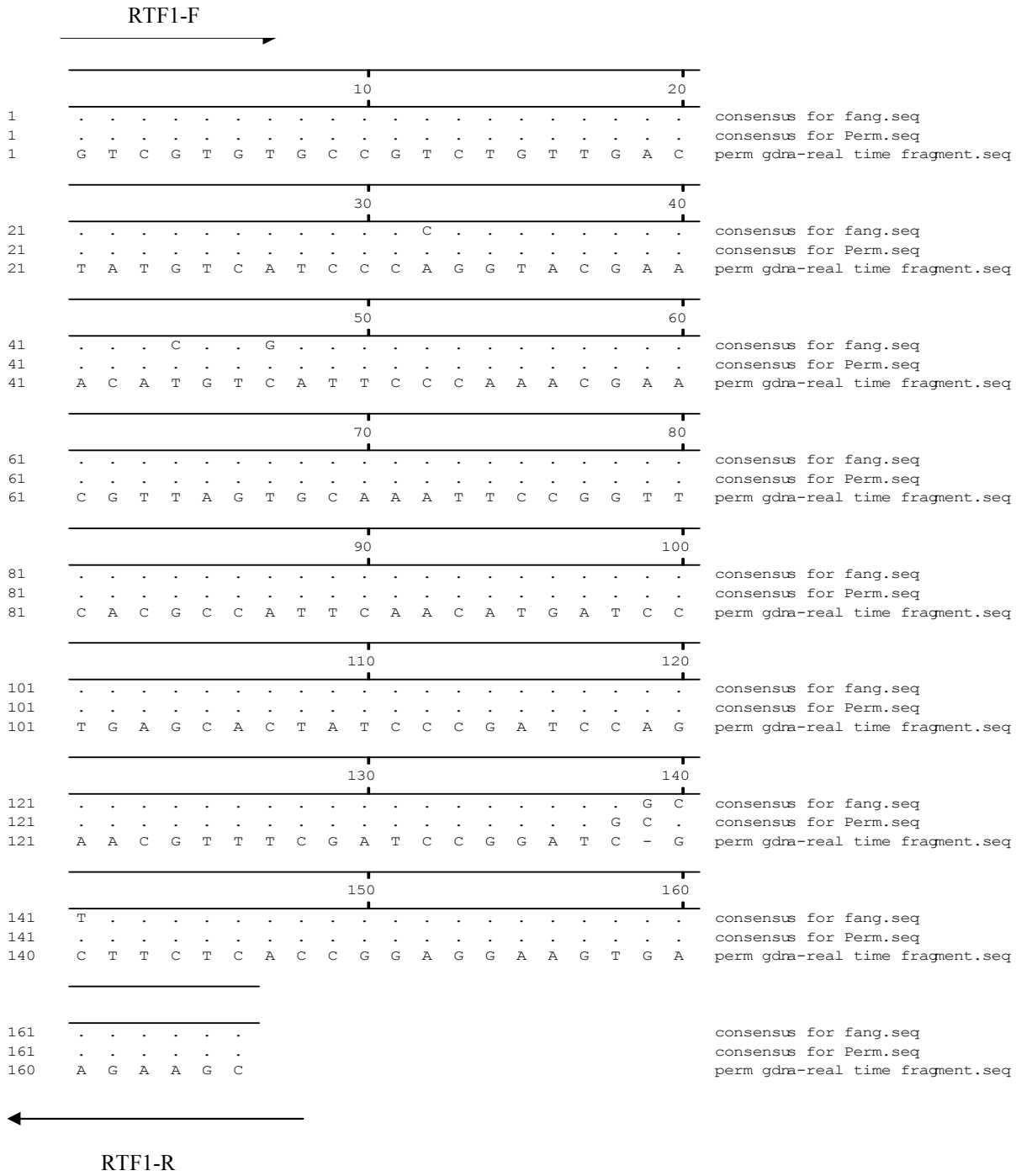


Fig: 30: Developmental expression of CYP6P9 and rsp7 genes in FUMOZ-R and FANG. PCR products of the real time assay described in Fig. 20 were analysed on a 1% (w/v) agarose gel and stained using EtBr. SL (3rd instar FANG larva), SP (FANG pupa), RL (3rd instar FUMOZ-R larva) and RP (FUMOZ-R pupa). The 300 bp band was amplified in the pupa of both FUMOZ-R and FANG.

Sequence analysis using BLAST (http://p450.antibes.inra.fr/) for the real time PCR products from FUMOZ-R and FANG revealed that the 170 bp band was identical to the CYP6P3 gene in *An. gambiae*. Real-time PCR fragments were sequenced to confirm that the correct gene was amplified. Three samples were sequenced for FUMOZ-R and 2 samples for FANG. The FUMOZ-R and FANG sequences were identical to CYP6P3 gene with a percent identity of 85 and 71 respectively. Pairwise alignment showed that the partial CYP6P9 gene from

FUMOZ-R and FANG were 96% identical to each other (Figs. 31A and B). Sequencing reactions for the 300 bp band from the pupa to confirm its identity did not yield good sequence results.

A



B

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FANG    VVCRLLTMSSPVRN-TFPNERCKFRFTPFNMILSTIPIQNVSIRIAFSPEEVKK 53
PERM    VVCRLLTMSSQVRNMSFPNERCKFRFTPFNMILSTIPIQNVSIRMRFSPEEVKK 54
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Fig. 31: A: Pairwise nucleotide sequence alignment of FUMOZ-R and FANG 170 bp fragment of the CYP6P9 gene. The amplified products after real time were purified and sequenced. Perm gDNA sequence was kindly supplied by T. S Matambo. The arrows indicate the CYP6P9 primers.

B: Predicted amino acid sequence alignment of FUMOZ-R and FANG 170 bp fragment. Identical or highly similar residues are shown in asterisks and colons. while the absence of a nucleotide at the specific position in the respective sequence is indicated by a gap.

4.3.5 Expression of CYP6P9 using hybridisation probe

Real time PCR was repeated using hybridisation probe chemistry to allow sequence specific detection by the probes and eliminate false positives due to non-specific product formation. Melt curve analysis revealed two peaks in the cDNA samples at 55 ^{o}C annealing temperature. When analysed on agarose gels, two fragments of 300 bp and the expected 170 bp were observed in FUMOZ-R and the 300 bp fragment in FANG (Fig. 32). At 60 ^{o}C annealing temperature, the 170 bp fragment was observed only in FUMOZ-R and not in FANG (Fig. 32).

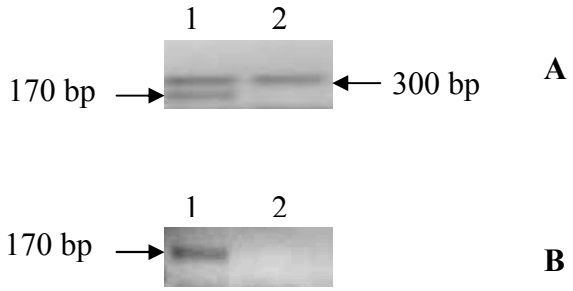

Fig. 32: QRT-PCR using hybridisation probes. (A) 55 ^{o}C: Lane 1: FUMOZ-R cDNA; Lane 2: FANG cDNA. (B) 60 ^{o}C: Lane 1: FUMOZ-R cDNA; Lane 2: FANG cDNA.

4.4 DISCUSSION

4.4.1 Expression analysis of the CYP6P9 gene in a resistant strain of *An. funestus*

4.4.1.1 Dot blot and northern blot analysis

The initial expression patterns of P450 genes in FUMOZ-R and FANG using cocktail probes specific for CYP4, CYP6 and CYP9 genes were carried out by dot blot analysis. The dot blot technique was found to be a fast and effective method of providing information on the expression of CYP genes in the FUMOZ-R and FANG. Unlike the northern blot analysis where the process takes two days, dot blot analysis can be carried out in one day. The choice to use 3-day old adult mosquitoes for expression studies was based on bioassay and biochemical assays.

Monooxygenase levels in FUMOZ-R were found to be comparatively high at this age (Hunt *et al.*, 2005). The rsp7 gene was used as a housekeeping gene in expression studies as the gene is constitutively expressed in *Anopheles* species (Salazar *et al*., 1993; Dimopoulos *et al*., 1999). Results from the analysis using cocktail probes showed only expression of CYP6 class in FUMOZ-R. The CYP4, CYP6 and CYP9 genes have been reported as over-expressed in other pyrethroid resistant insects (Pittendrigh *et al.,* 1997; Rose *et al*., 1997; Pridgeon *et al*., 2003; Nikou *et al*., 2003).

Although no signals were visualised on blots containing CYP4 and CYP9 probes, the genes that comprised the cocktail probes were a significant representation of the two P450 families. This is based on the total number of P450 genes that have been isolated in the two P450 families in *An. funestus* (Amenya *et al*., 2005). Furthermore, because expression was not detected in 12 CYP4 and 5 CYP9 genes, it is suggested that their transcripts are rare and expressed at very low levels in FUMOZ-R and FANG. This however, does not mean that other genes within these two families are not involved or over-expressed (Ranson *et al*., personal communication). The observation that a CYP6 class was expressed in a pyrethroid resistant strain of *An. funestus* contributes to the literature that has reported over-expression in CYP6 P450s (Feyereisen *et al*., 1995, Liu and Scott, 1998; Kasai and Scott, 2000; Nikou *et al*., 2003; Rongnoparut *et al*, 2003; Rodpradit *et al*., 2005). For instance, Rongnoparut *et al*, (2003) showed that deltamethrin resistance in *An. albimanus* was due to increased levels of P450 activity resulting from elevated expression of CYP6AA2. The lack of signals on FANG RNA using

CYP6 probes could be due to low levels of expression of the genes and increasing the initial starting concentration of RNA will elliminate this problem.

After the identification that a CYP6 class was expressed in FUMOZ-R, it was important to identify the actual gene that was over-expressed. This was achieved by individual probing of the genes comprising of the CYP6 cocktail on dot blots. Optimisation of the RNA spotted on the dot blot was necessary in determining the concentration at which a signal was visualised in FANG. This was due to the assumption that at higher concentrations of RNA, a signal would be detected in FANG. Various RNA concentrations were investigated for their ability to present a signal in FANG. A gradual increase in blot intensity of FUMOZ-R was observed at RNA concentrations above 25 μg with subtle increase in the FANG. Detectable signals in the FANG dilutions were achieved when the starting RNA concentration was 50 μg (data not shown). The excessive background observed on spots (spot 4 and 5) on the membrane probed with rsp7 (Fig. 17) might have been due to non-specific hybridisation. This is common in membranes that are stripped and re-hybridised due to the membrane drying during stripping procedure and increase in the wash temperature. Evaporation of the wash solution used to strip probes, results in drying of salts on the membrane causing a blotchy background (Fig. 17). However, subsequent QPCR results have clearly shown that the rsp7 gene is expressed equally between the two strains (see section 4.4.1.3).

Results revealed the over-expression of the CYP6P9 gene, which is an ortholog of CYP6P3 gene in *An. gambiae* (Amenya *et al*., 2005). In *An. gambiae*, a CYP6Z1

gene was found to be over-expressed in a pyrethroid resistant strain from Kenya (Nikou *et al*., 2003). However, the same gene was not over-expressed in *An. funestus* (data not shown). Microarray analysis between the resistant and susceptible lines of *An. gambiae* did show that other P450's were differentially expressed, but none were CYP6P3 (David *et al*., 2005). This shows that there is a significant variation in the genes suspected to be involved in pyrethroid resistance between these two *Anopheles* species. CYP6P9 expression was found to be higher in the FUMOZ-R strain compared with the FANG strain. Similarly, differences in expression of P450 genes between a resistant and a susceptible strain have also been reported in pyrethroid resistant tobacco budworm *H. virescens* and *An. gambiae* (Rose *et al*., 1997; Nikou *et al.*, 2003). mRNA levels of CYP6Z1 expression in *An. gambiae* and CYP9A1 in *H. virescens* were higher in the resistant strains than in the susceptible strains.

4.4.1.2 Semi-quantitative expression of CYP6P9 gene

In general, semi-quantitative PCR using both primer pairs revealed a higher expression of CYP6P9 gene in the FUMOZ-R strain compared with the FANG strain. This observation is based on the band intensities between the two strains. CYP6P9 gene amplification using RTF2 primer pairs was expressed at a lower level in the egg compared to the larva and pupa of FUMOZ-R. In FANG, a similar observation was made in which expression of CYP6P9 was undetectable in the eggs but expressed in the larva and pupa. It is possible that the CYP6P9 gene is present in eggs of both FUMOZ-R and FANG but due to the low

sensitivity of semi-quantitative PCR method, the bands are undetectable on agarose gels. The expression of CYP6P9 gene in adult FUMOZ-R was also found to be higher than in FANG adults.

The discrepancy reflected in FUMOZ-R egg and adults using the two different primer pairs could be due to primer design, PCR efficiency and optimisation conditions.

There are slight differences in the band intensities of the rsp7 fragments. This is most likely an artifact of conventional PCR due to the fact that the non-exponential plateau phase are visualised on agarose gels. Making this method less accurate. The main advantage of using Quantitative real-time PCR is that the amplicons are visualised during the exponential phase, making it more sensitive for expression studies.

The choice to use rsp7 as an internal standard was based on previous experiments with similar experimental conditions in *An. gambiae* (Dimopoulos *et al*., 1999; Nikou *et al*., 2003). It was demonstrated that this gene is also suitable to use for *An. funestus* expression studies (see section 4.4.1.3 for additional support). The partial rsp7 gene sequenced in *An. funestus* is 92% identical to *An. gambiae* rsp7 (data not shown).

The semi-quantitative results are in agreement with the bioassays in which resistance was high in the larvae and adults of FUMOZ-R (B. Brooke personal

communication). This selection for the resistance phenotype gene is thought to take place in the larval stage and carried through to the adult stages. This is mainly due to larval breeding habitats that are generally in close proximity to agricultural activities. Insecticides used for agricultural pest control leach into the breeding habitats and therefore exert selection pressure on the larvae. The dependency and extensive usage of pyrethroids against the cotton bollworm *H. armigera* for example, have imposed severe selective pressure resulting in higher levels of resistance in the larvae (Duraimurugan and Regupathy, 2005). Pridgeon *et al*., (2003) hypothesised that the over-expression of CYP4G19 in nymph and adults of the resistant strain *B. germanica* is an adaptive ability to metabolise xenobiotics both at nymph and adult life stages. It is therefore not unique that CYP6P9 was expressed in both the larval and adult stages of FUMOZ-R.

Although gene expression patterns are known to provide clues on the biological role of a gene in the life stages of insects (Kasai and Tomita, 2003), the low sensitivity of semi-quantitative PCR results in limited information being obtained using this method. It is however, cost effective to use before embarking on more costly assays such are Quantitative real-time PCR. The semi-quantitative PCR technique gave an indication of the pattern of expression of CYP6P9 gene in the life stages of both mosquito strains. It was necessary to confirm these results using a more sensitive and accurate technique such as quantitative real time PCR.

4.4.1.3 Quantitative real time PCR of CYP6P9 gene

CYP6P9 expression using real time PCR suggests that this P450 is associated with pyrethroid resistance in FUMOZ-R. From the quantitative real time analysis, there was no significant difference in CYP6P9 gene expression between FUMOZ-R males and females at either 3-day old or 14-day old. Similarly, there was no difference in the intensities of the real time PCR products in both sexes (Fig. 29) although CYP6P9 expression was 4.5 fold higher in the 3-day old males than the females of the same age. These results correlate with that of Hunt *et al*., (2005) where there was no significant difference in monooxygenase levels in the males and females of FUMOZ-R. Furthermore, bioassays conducted on FUMOZ-R showed that resistance increased by day 4 and decreased significantly by day 10 (Hunt *et al*., 2005). CYP6P9 expression is therefore not sex specific as has been shown in P450s involved in the metabolism of sex hormones (Kasai and Tomita, 2003).

Efforts to try and eliminate primer dimers in FANG cDNA by optimising primer concentrations and annealing temperature were without success. In real time PCR, low copy number of target DNA is associated with the formation of primer dimers, reduced fluorescence and non-specific products (Teo *et al*., 2002; Peters *et al*., 2004). The presence of primer dimers can lead to anomalous quantification values such as early or later C_T values. No primer dimers were detected in FUMOZ-R cDNAs on the melt curves or on the PCR products that were analysed on agarose gels. The high similarity of the primer binding sites on the CYP6P9

gene in both FUMOZ-R and FANG ruled out the possibility that the primers were not specific enough (Fig. 31). The percent identity of the partial CYP6P9 amplified in both FUMOZ-R and FANG showed amino acid differences between these two strains (Fig. 31). Sequencing of the full-length CYP6P9 gene from both FUMOZ-R and FANG will be important to establish the importance and relevance of these mutations in the enzyme.

Threshold values obtained from pupa were not used to estimate CYP6P9 copy numbers as a result of the amplification of the 300 bp band. Semi-quantitative PCR revealed only the expected 170 bp band. A possible explanation is that due to the sensitivity of quantitative real time PCR, a gene with a similar primer binding site to CYP6P9 was amplified in the pupa using RTF1 primer pairs. In holometabolous insects, the pupal stage is a resting stage characterised by nonfeeding and up or down regulation of genes (Denlinger, 2002). In *An. gambiae*, a CYP6Z2 gene was expressed in the larva and adult stage and not in the pupal stage (Nikou *et al*., 2003). Secondly, the amplification of the 300 bp band may be due to the difference in the PCR conditions of real time and semi-quantitative methods (Figs. 30 and 32). This assumption is based on the fact that a 170 bp band was amplified in semi-quantitative PCR at $MgCl_2$ concentration of 1.5 mM while in real time PCR, the $MgCl_2$ concentration was 6 mM in the PCR reaction mix. Although, real time PCR has been shown to require higher concentrations of $MgCl_2$ for optimal results (Boeckman *et al*., 2000), the higher amount of $MgCl_2$ could have lead to the preferential amplification of another gene. Further investigation regarding this fragment is necessary.

The differences observed using hybridisation probe chemistry at different annealing temperatures with FUMOZ-R and FANG showed that results obtained using SYBR Green and hybridisation probe chemistries cannot be compared in this particular circumstance. The hybridisation chemistry is considered a better method compared to SYBR Green chemistry, however, the former was found to require more optimisation. In this case, it was not possible to carry out adequate optimisation using hybridisation chemistry due to lack of time, resources and expenses. The specific primers used in the real time PCR were designed from the partial gene of CYP6P9 isolated in Chapter 3 (Fig. 4). It will be essential that once the full-length gene of CYP6P9 is obtained, gene specific primers are re-designed from other regions and the quantitative PCR repeated.

4.4.2 Genetic basis of resistance

Resistance in insects is considered to be a consequence of rapid Darwinian selection in natural populations as a result of strong selection pressure (Soderlund and Bloomquist, 1989; Scott and Kasai, 2004). Current understanding of the evolution of insecticide resistance results from mutation of the target sites (*Rdl*, *kdr* or *super-kdr*) or from overexpression of GSTs, esterases and P450s. The genetic basis of resistance has been studied in *An. gambiae*, houseflies and *Drosophila* (Ranson *et al*., 2000b; Sabourault *et al*., 2001; Darbon *et al.*, 2002; Scott and Kasai, 2004). However, this is yet to be recorded in *An. funestus*.. Preliminary results on crosses between the pyrethroid resistant strain and the susceptible strain have revealed the resistant gene as incompletely dorminant and

monofactorial (P. Okoye, personal communication). Fitness studies have revealed very little difference between these strains, however it was found that the resistant females would lay eggs more frequent than the susceptible counter part. It is unclear whether this phenomenon is due the difference in the resistance status or due to the fact that these strains originate from different geographical areas (B. Brooke personal communication).

Various studies have been conducted on resistant phenotypes to understand the genetic mechanism involved in resistance. These studies had focused on quantitative studies to pin point the location of these genes on the genome and performing crossing experiments. Scott and Kasai (2004) showed that resistance in a strain of housefly (NG98) from Georgia was due to *kdr* on autosome 3, while monooxygenase-mediated resistance was located on autosomes 1, 2 and 5. His results indicate that genes that evolve to produce monooxygenase-mediated resistance to permethrin are different between housefly populations exhibiting some degree of plasticity in response to selection. In the multi-resistant Rutgers strain of houseflies, overproduction of CYP6A1 that metabolises diazinon was linked to a loss of function in the chromosome 2 aliesterase (MdαE7) gene (Sabourault *et al*., 2001). The Gly137 to Asp point mutation in the αE7 esterase gene or a deletion of the locus was shown to confer resistance and overproduction of the CYP6A1 protein (Sabourault *et al*., 2001).

Genetic crosses of ZAN/U, a DDT resistant strain of *An. gambiae* from Zanzibar showed that resistance was autosomal and semi-dominant (Ranson *et al*., 2000b).

Genetic mapping of the first locus affecting this resistance was found to lie on chromosome 3 exhibiting a recessive effect with respect to susceptibility (Ranson *et al*., 2000b). The second locus, rtd2 was found to lie on chromosome 2L and exhibits a genetic effect. In *Drosophila*, Darbon *et al.* (2002) used a microarray approach in a DDT resistant *Drosophila* strain. They found that the resistance and upregulation of P450's are associated with a single Cyp6g1 allele. The allele was characterised by the insertion of an *Accord* transposable element in the 5' end of the Cyp6g1 gene.

CHAPTER 5

cDNA LIBRARY CONSTRUCTION

5.1 INTRODUCTION

Scott, (1999) reported that increased expression of P450 genes could be due to mechanisms such as: (a) increased transcription (b) gene amplification (c) stabilisation of the mRNA (d) or a change in the protein resulting in greater catalytic activity. Most of these mechanisms can be studied with the availability of the full-length cDNA clones of the over-expressed P450. The screening of cDNA libraries is a widely used method to obtain a full-length cDNA clone of a new protein. For instance, the use of partial P450 fragments has been employed in screening cDNA libraries in order to isolate P450s with a specific function (Xiao-Ping and Hobbs, 1995). In Chapter 4 of this thesis, partial P450 fragments isolated from *An. funestus* were used to identify the over-expressed gene in the resistant FUMOZ-R strain of *An. funestus*. The CYP6P9 gene was over-expressed in FUMOZ-R when compared to the susceptible FANG strain.

In as much as the present chapter does not answer the above questions, the constructed *An. funestus* cDNA library and the optimised screening conditions using partial fragments of CYP4, CYP6 and CYP9 genes, lay a solid foundation for future studies on the CYP6P9 gene.

5.2 MATERIALS AND METHODS

Anopheles funestus cDNA library was constructed in λTriplEx2 vector as presented in Chapter 2. Optimisation of screening procedures of the library using *An. funestus* P450 DIG-labeled probes to obtain full-length P450 cDNA sequences, was carried out as described in Sambrook *et al*., (1989) with modifications as described in Appendix A. Positively identified clones were sequenced using 5' and 3' pTriplEx2 sequencing primers.

5.3 RESULTS

5.3.1 *Anopheles funestus* cDNA library

The cDNA synthesised from Poly $(A)^+$ mRNA was observed as a smear with a 900 bp band for the control poly $(A)^+$ human placenta RNA and 1500 bp band for the *An. funestus* poly $(A)^+$ RNA on a 1% (w/v) agarose gel (Fig. 33). Using the SMART protocol, a bright band with a smear is an indication of a successful Long Distance (LD) PCR (Zhu *et al*., 2001). A smear from approximately 4 Kb (close to the origin/well) to approximately 300 bp was observed. This showed that DNA fragments with high molecular weight were amplified and that one should have full-length cDNA sequences in the amplified product. This also signified that starting RNA was not degraded or impure. The bright fragment represents abundant mRNA in the mosquito. This fragment was neither too faint nor too bright when compared to the intensity of the size-marker thus indicating that the

cycling conditions did not need optimisation. The cDNA obtained was used as template in the construction of the cDNA library. The titer of the unamplified and the amplified library was calculated to be 4.0 x 10^7 pfu/ml and 4.9 x10^8 pfu/ml respectively. A transformation efficiency of 98% was achieved.

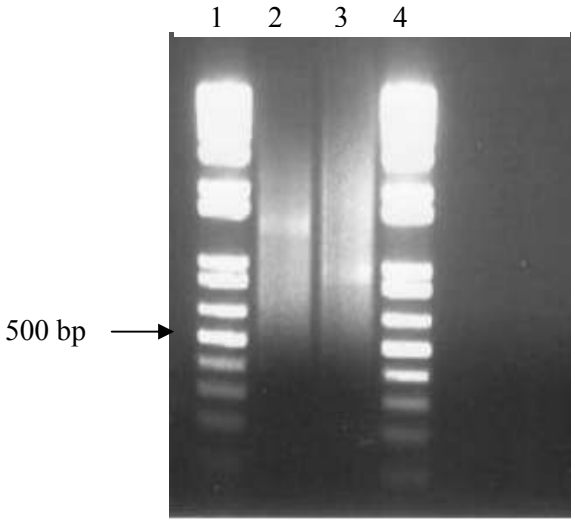

Fig. 33: An agarose gel profile of double stranded cDNA synthesised by LD-PCR method using SMART^{TM} cDNA library construction kit.
Lanes 1 and 4: 1 kb ladder (Invitrogen); Lane 2: *An. funestus* cDNA; Lane 3: control poly $(A)^+$ human placenta cDNA.

5.3.2 Optimisation of cDNA library screening using P450 DIG-labeled probes

The cDNA library was screened using cocktail probes consisting of partial CYP4, CYP6 and CYP9 P450 genes. After screening for inserts, thirty positive clones of

approximately 1300 bp were selected and the plasmids purified. These clones were chosen to determine their nucleotide sequences identity.

From a total of 30 clones originally collected for sequencing, only 12 of these produced analysable sequence results. Alignment results using SeqMan (Lasergene 6) revealed that 5 of these clones were similar and were grouped into one contig (data not shown). Using the UTResource programme (http://www.bighost.area.ba.cnr.it/BIG/UTRHome) it was concluded that one of these clones (Pb-6) was within the UTR of an mRNA molecule from the Honey bee (3AME000220). Fig. 34 represents an annotation of a UTR of the cDNA library clone (Pb-6). Pb-6 5' UTR was 95% identical to 3AME000220. This UTR reference contains the 3'UTR in *Apis mellifera* AmGluRB mRNA for the metabotropic glutamate receptor. However, when the same sequence (181bp in length) was submitted to NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) the sequence showed 100% homology (for 117bp/180bp) with *An. funestus* 16S ribosomal gene located on the mitochondrial DNA (accession number: DQ287368.1). Figure 35 is an alignment between the partial 16S ribosomal gene (DQ287368.1) and Pb-6, showing the high degree of homology within this sequence.

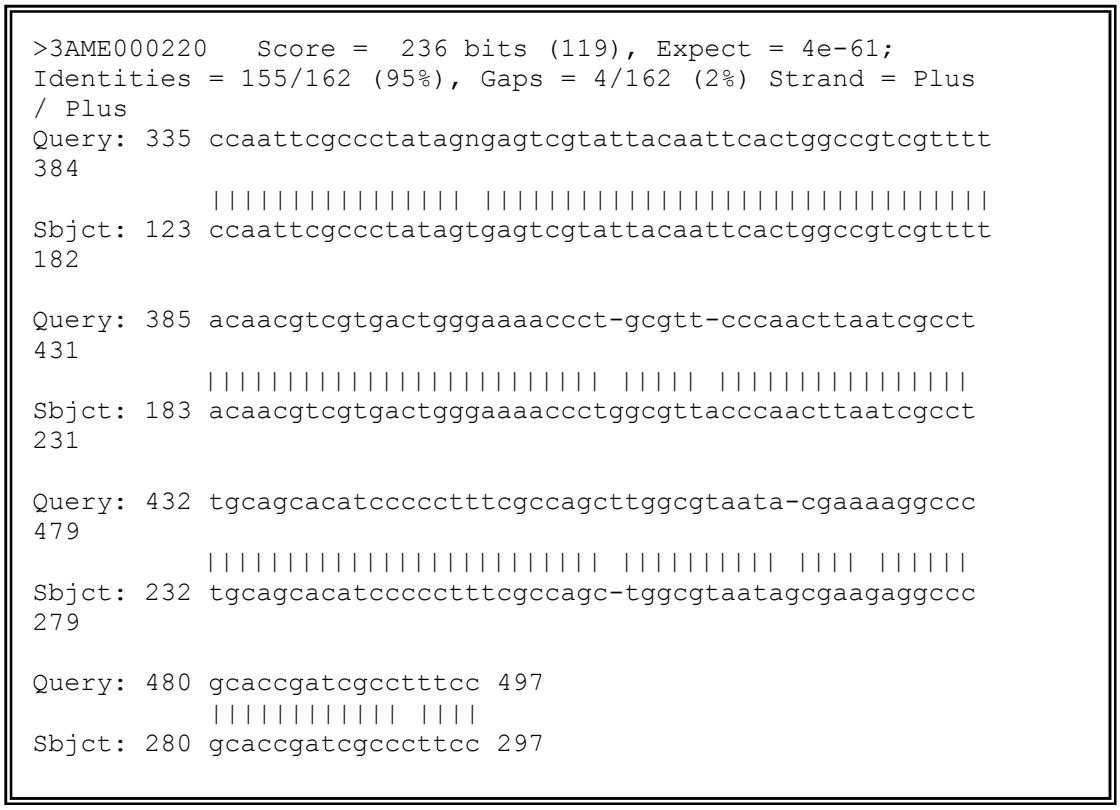

Fig. 34: Clone Pb-6 annotation of the 5' UTR. Query: Pb-6 sequence; Sbjct: 3AME000220.

Sequence query using NCBI Blast further revealed one clone with homology to *Aedes aegypti* Cytochrome C subunit 1, as well as a number of clones with unknown origin. These however, showed homology with *An. gambiae* full-length cDNA sequences although the particular cDNA sequences have not yet been annotated. Three more clones produced no specific BLAST result and it is difficult to speculate what these might be.

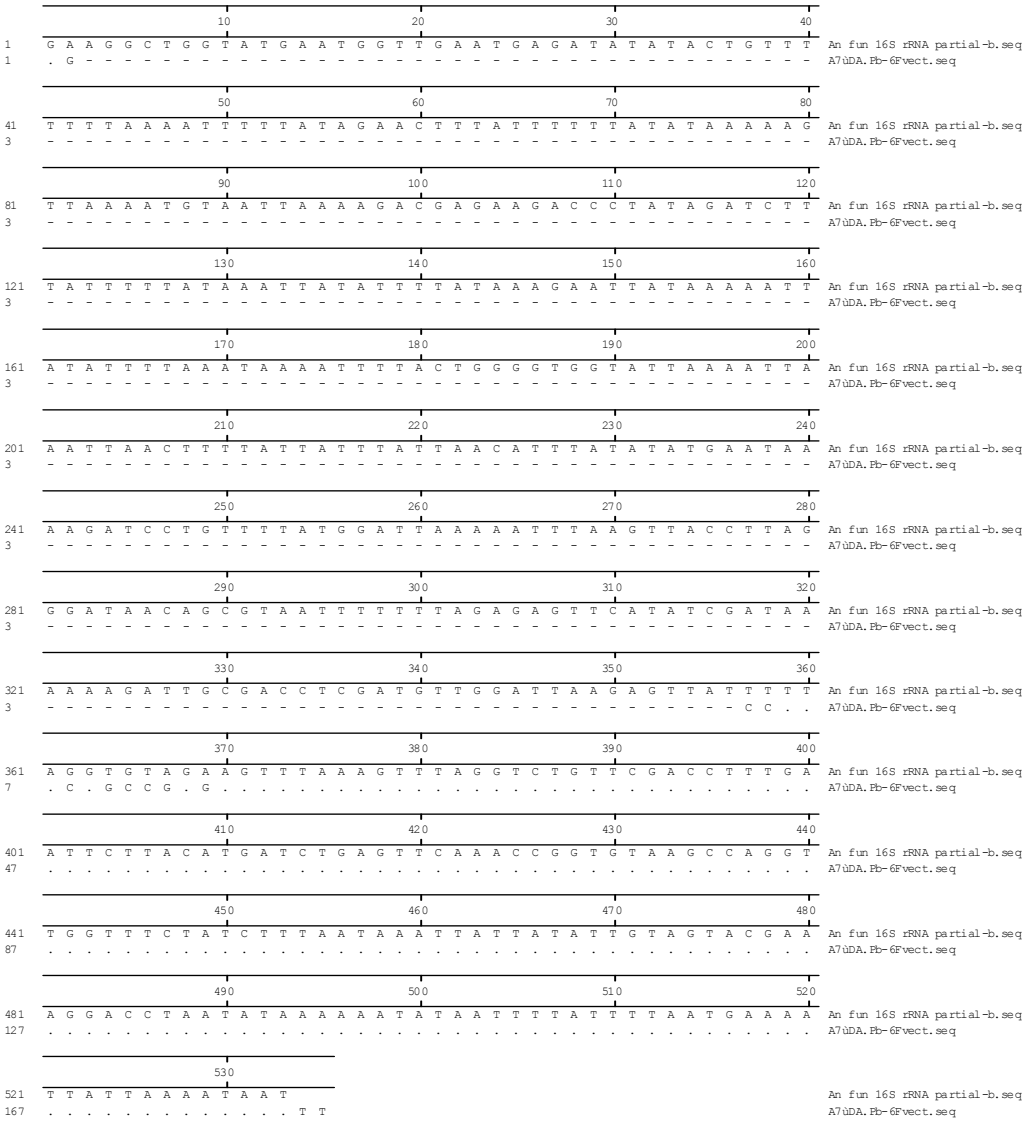

Figure 35: Sequence alignment between cDNA clone Pb-6 and partial fragment of the 16S ribosomal gene, located on the mitochondrial genome, from *An. funestus*. (-) indicates the absence of a nucleotide at that specific position, while (.) indicate the same nucleotide at that specific position.

5.4 DISCUSSION

5.4.1 Construction of *An. funestus* cDNA library

Anopheles funestus cDNA library constructed in λTriplEx2 vector was successful based on the capacity of the library titer and the transformation efficiency that was achieved after library amplification. According to Sambrook *et al*., (1989), a library constructed from a fragment of 4 kb should contain at least 1.0 x 10^7 independent recombination to have a chance of including desired sequences. The average inserts of the recombinants (1.3 kb) in the cDNA library were also optimal (Zhu *et al.,* 2001). The SMART technology that was employed in constructing the *An. funestus* cDNA library avoided bias of cloning short cDNA products which is common in PCR-based constructed cDNA libraries. This was achieved through size fractionation using CHROMA SPIN columns. The method also ensured that the library contained full-length cDNAs that preserved the complete 5' terminal sequence of mRNA (Barnes, 1994). As a result, the *An. funestus* cDNA library could be useful in finding a cDNA clone derived from low abundance mRNA.

5.4.2 Optimisation of screening conditions using P450 probes

The sequencing results discussed below were based on a single reaction for each clone and is inconclusive as re-sequencing of these clones is necessary. This will confirm the identity of these sequences and rule out sequencing errors.

Optimisation of the screening procedures for the library was carried out, however the fact that no specific P450 sequences were isolated indicates that the stringency used was not high enough. This will be important in targeting a specific gene sequence using specific probes and avoiding false-positives. The temperature at which the hybridisation is carried out and the ionic strengths of the buffers are critical and may influence hybridisation using nucleic acids (Isoe *et al.,* 2005). During the sequence cycling steps, it was found that higher plasmid concentrations (> 400 ng) at an annealing temperature of 50 ^{o}C gave better results. The fact that several clones sequenced produced BLAST search results with *An. gambiae* full-length cDNA clones is also encouraging in that the library contains full-length sequences although the sequences do not contain any annotations to date.

UTRs are good indicators of a full-length sequence and are involved in many post-translational regulatory pathways in prokaryotes and eukaryotes (Mignone *et al*., 2002). After removing the vector sequences, UTR length for sequences analysed was found to be between 20 – 162 bp although the length of 5' and 3' UTRs in most organisms can range from dozens to thousands of nucleotides within species (Pesole *et al*., 2001; Mignone *et al*., 2002). The contradictory results using two independent search engines, BLAST and UTResource, is problematic. The fact that the preliminary sequence data analysis did not reveal the translation initiation sequence (ATG) or the translation stop codon (TGA) of the genes, might indicate that Pb-6 does not contain a UTR as was found with the

UTResource programme. However, further investigations are needed before any conclusion can be drawn for that specific clone.

In an attempt to isolate full-length P450 genes it is recommended that the PCR-based method for screening the library be used alongside filter hybridisation. The PCR-based screening method is sensitive and less time consuming as it allows for easy identification of a particular clone in a portion of the library. The method also provides a high degree of stringency. Once a positive clone is identified by PCR, the library lysate from which the clone was obtained can be plated and the results confirmed by the traditional hybridisation method. As a result of the high identity between *An. funestus* and *An. gambiae* P450 genes (Amenya *et al*., 2005), the presently constructed *An. gambiae* microarray detox chip will be useful (David *et al*., 2005). The detox chip contains genes putatively involved in insecticide metabolism and could be used to monitor expression of P450 genes in *An. funestus*.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

In the physical environment, insects are constantly under assault by chemicals such as insecticides and pesticides. P450s among other things provide insects the ability to breakdown and detoxify poisonous substance.

1. This study has revealed very high P450 sequence identity and similarity between two species (*An. funestus* and *An. gambiae*) that in evolutionary terms are far apart.
2. Using dot and northern blot analysis, CYP6P9 gene expression was higher in the resistant, FUMOZ-R strain than in the susceptible, FANG strain.
3. CYP6P9 was developmentally regulated. Using semi-quantitative PCR, revealed undetectable levels of CYP6P9 in egg, higher in larva compared to pupa in both FUMOZ-R and FANG strains.
4. CYP6P9 was expressed in both males and females of FUMOZ-R. Expression of CYP6P9 was 4.5-fold higher in 3-day old FUMOZ-R males than females of the same age and 3.5-fold higher in the 14-day old males than 14-day old females. However, the expression was not statistically significant. CYP6P9 expression is therefore not sex specific.
5. The primer dimer detection on FANG cDNAs after melt curve analysis suggests the presence of low copy number of the CYP6P9.

6. The detection of the 300 bp band in the pupa using quantitative real time PCR technique shows that the method is more sensitive compared to semi-quantitative PCR.

Based on the consistency of the data obtained from the expression of CYP6P9, it is possible that this gene may be related to the development of pyrethroid resistance in FUMOZ-R. Metabolic differences between these two *An. funestus* strains may be investigated in future through heterologous recombinant protein expression and enzyme assays. A way forward to this has been established through the construction of *An. funestus* cDNA library. Consequently, functional studies on CYP6P9 could be used to determine whether FUMOZ-R produces a functionally efficient cytochrome P450 that speeds up the metabolism of pyrethroids. This will further improve the understanding and give insights into the molecular basis of P450-mediated resistance in *An. funestus* species.

APPENDIX A

STANDARD PROCEDURES

A.1 RNA quantification

RNA quantification was carried out as described by Sambrook *et al*., (1989) Readings were taken at 260 nm and 280 nm. A 260 nm reading was used to calculate the concentration of nucleic acids in the sample. Calculations were carried out according to the following formula:

1 OD_{260} = 50 μg/ml DNA

1 OD_{280} = 40 μg/ml RNA

The ratio between OD_{260}/OD_{280} provided an estimate of the purity of the nucleic acid. Pure DNA and RNA preparations have OD_{260}/OD_{280} values between 0.8 and 2.0.

A.2 Elution of DNA from agarose gel

Elution of DNA was carried out using QIAquick^{TM} PCR purification kit (Qiagen, cat. no. 28106) according to manufacturer's protocol.

After degenerate PCR, each DNA band was excised from the agarose gel using a clean, sharp scapel blade and placed in 1.5 ml microcentrifuge tube. Purification was carried out separately for each CYP family. The gel slice was weighed and 3 vol of buffer QG added to 1 vol of the gel (100 mg ~ 100 μl). The tube was incubated at 50 ^{o}C until the gel slice completely dissolved. The gel was dissolved by vortexing the tube every 2 – 3 min during incubation. On complete dissolution,

the colour of the mixture was yellow. One vol of isopropanol was added and the tube contents mixed well. A QIAquick spin column was placed in a provided 2-ml collection tube. The sample was applied to the column to let DNA bind to it and centrifuged (704 x g, 1 min, 25 ^{o}C). The flow-through was discarded and the column placed on the same tube. To wash the column, 0.75 ml of buffer PE was added and the column in the tube centrifuged (704 x g, 1 min, 25 ^{o}C). The flow-through was discarded and the column re-centrifuged (704 x g, 1 min, 25 ^{o}C). The column was placed in a clean 1.5 ml microcentrifuge tube. Thirty µl of sterile distilled water was added to the centre of the column, left to stand for 3 min and centrifuged (13,226 x g, 1 min, 25 ^{o}C).

A.3 Concentration of DNA

This procedure was carried out when the DNA concentration in ng/µl could not be quantified using the Genequant *pro* Spectrophotometer at dilutions of 1:50 or 1:100. Either isopropanol or ethanol precipitation methods were used (Sambrook *et al*., 1989).

A.3.1 Isopropanol precipitation

To every 1 vol of DNA solution, ½ vol of 7.5M ammonium acetate or 1/10 vol 3M NaAc, pH 4.8 and 0.7 vol of Isopropanol were added and mixed. The mixture was kept for 15 min at room temperature and centrifuged (13,226 x g, 15 min, 25 ^{o}C). The supernatant fraction was discarded and the pellet washed in 1 vol of 75%

ethanol and re-centrifuged (4,402 x g, 5 min, 25 ^{o}C). The pellet was dried and re-suspended in an appropriate amount of sterile distilled water.

A.3.2 Ethanol precipitation

To every 1 vol of DNA solution, 1/10 vol 3M NaAc, pH 4.8 and 2.5 vol 100% chilled ethanol were added. The mixture was incubated at -20 ^{o}C for 30 min and the DNA recovered by centrifugation (13,226 x g, 30 min, 4 ^{o}C). The pellet obtained was washed in 1vol of 75 % ethanol and re-centrifuged (13,226 x g, 5 min, 25 ^{o}C). The pellet was dried and re-suspended in an appropriate amount of sterile distilled water.

A.4 Preparation of competent cells

Competent cells were prepared using Hanahan's protocol (1985). A single colony of *E. coli* strains JM109 bacterial cells as inoculated into 20 ml Luria Bertini (LB) broth (10 g/l Bacto tryptose, 5 g/l Bacto yeast, 5 g/l NaCl. pH 7.0), grown at 37 ^{o}C with moderate shaking (200 rpm) to an OD_{600} = 0.2 to 0.8. One ml of the culture was inoculated into a sterile 250 ml flask containing 400 ml Psi broth (5 g/l Bacto yeast, 20 g/l Bacto tryptose, 5 g/l Mg_2SO_4, pH 7.6) and incubated at 37 ^{o}C with moderate shaking (200 rpm). As soon as the bacteria reached an OD_{600} = 0.6, the flask was put on ice to terminate the growth of bacterial cell. The bacteria suspension was centrifuged (1252 x g, 15 min, 4 ^{o}C). The supernatant fraction was discarded and the bacteria pellet re-suspended carefully in 100 ml TfbI (TfbI/200 ml: 30 mM KOH, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 15 % v/v

glycerol, pH 5.8) and kept on ice for 15 min. The bacteria suspension was re-centrifuged (1252 x g, 15 min, 4 ^{o}C), re-suspended in Tfb II (TfbII/100 ml: 10 mM MOPS, 75 mM $CaCl_2$ 10 mM $RbCl_2$, 15% v/v glycerol, pH 6.5) and aliquoted (200 µl aliquots) into ice-cold 0.2 ml microcentrifuge tubes. The aliquots were then frozen in liquid nitrogen and stored at –70 ^{o}C.

Competency of the cells was assessed using 10 ng pGEM® -T easy vector (Promega**,** cat. no. A1360) to transform 100 µl of competent cells. Luria Bertini agar (10 g/l Bacto tryptose, 5 g/l Bacto yeast, 5 g/l NaCl pH 7, 15g/l Bacto agar) plates containing Ampicillin (125 µg/ml) were prepared. Hundred µl of Isopropyl β-D-thiogalactopyranoside (IPTG: 0.1 M) and 40 µl of 5-bromo-4-chloro-3-indolyl-β-galactosidase (Xgal: 50 mg/ml) were spread onto LB agar Ampicillin treated plates. Aliquots (1, 10, 25 µl) of the transformation culture were spread on the plates and incubated at 37 ^{o}C overnight. Transformation efficiency (number of transformants per µg DNA) was assessed as a ratio of the recombinant (white colonies) to non- recombinant cells (blue colonies). When the total recombinants on the plate were above 80%, the cells were considered competent.

A.5 Ligation and transformation of PCR products

Purified PCR products of CYP4, CY6 and CYP9 were ligated into pGEM® -T easy vector (Promega**,** cat. no. A1360) according to manufacturer's instructions with modifications. Briefly, ten µl ligation reaction consisting of 5 µl of 2X rapid ligation buffer, 400 units of T4 DNA ligase (400 units/µl), 50 ng of pGEM® - T

easy vector (50 ng/μl), 50 ng of cleaned PCR product and deionised water was incubated overnight at 4 ^{o}C. A control reaction was set up to determine the ligation efficiency using control DNA (Promega**,** cat. no. A1360). To 10 μl of the ligation reaction, 50 μl of JM109 cells were added and incubated on ice for 30 min. The cells were heat shocked at 42 ^{o}C for 2 s and the tubes placed on ice for 2 min. Five hundred μl of NZY broth (10 g/l NZ amine, 5 g/l Bacto yeast, 5 g/l NaCl) was added to the mixture and incubated at 37 ^{o}C for 1 h. The mixture was briefly centrifuged and the pellet re-suspended in 200 μl of NZY broth. Luria Bertini plates were prepared as described in section 2.5. The culture (50 μl) was evenly spread onto the plates and incubated at 37 ^{o}C overnight. White colonies were picked and screened by PCR to confirm size of inserts.

A.6 Single Strand Conformation Polymorphism (SSCP)

To minimise repeated sequencing of identical clones, the selected cloned products were further analysed by SSCP as described by Koekemoer *et al*. (1999). Silver staining was carried out according to Budowle *et al*. (1991). Briefly, the gel was placed in a clean tray containing 10% ethanol for 10 min. One percent nitric acid was used to oxidise the gel for 6 min. The gel was rinsed twice in distilled water and stained using 0.012 M $AgNO_3$ for 30 min. After staining, the gel was rinsed in distilled water and placed in reducing solution (0.28 M $NaCO_3$, 0.019% Formalin) until the desired intensity was achieved. The reaction was stopped using 10% glacial acetic acid and the gel air dried on a filter paper. Clones that showed

unique migration patterns were grown overnight in 5 ml LB culture and purified by alkaline lysis technique (see section A.8 for details).

A.7 Screening of inserts using PCR

Abundant clones were screened by PCR using Sp6 (5'-TAC GAT TTA GGT GAC ACT ATA G-3') and T7 (5'-GTA ATA CGA CTC ACT ATA GGG-3') primers. Twenty five µl PCR reaction mixture containing 2.5 µl 10X PCR buffer, 5 µM each of the primer, 10 mM dNTPs, 1.5 mM $MgCl_2$ and 2.5 units Takara Taq^{TM} polymerase was prepared. The PCR conditions were 94 ^{o}C for 1 min followed by 32 cycles at 94 ^{o}C for 1 min, 55 ^{o}C for 30 s and 72 ^{o}C for 1 min. This was followed by a final extension of 72 ^{o}C for 10 min. PCR products were electrophoresed on 1.5 % (w/v) agarose gel in 1X TAE buffer (including 10 mg/ml EtBr). Clones that showed insert size above 400 bp for CYP4, CYP6 and CYP9 classes were streaked onto another LB-agar Ampicillin plate and subjected to single stand conformation polymorphism (SSCP).

A.8 Small scale plasmid purification (Mini-prep) by alkaline lysis technique

This procedure is based on alkaline lysis of bacterial cells (Birnboim and Doly, 1979) followed by purification of the DNA by ion-exchange chromatography and was carried out according to manufacturer's recommendations for the Qiagen plasmid mini kit.

A single colony was picked and inoculated in 5 ml of LB medium. The culture was incubated at 37 ^{o}C overnight with vigorous shaking (200 rpm). Following overnight incubation, the bacterial cells were harvested by centrifugation (13,226 x g, 15 min, 4 ^{o}C) and re-suspended in 300 ml of buffer P1. The mixture was lysed using 300 ml of chilled buffer P2, mixed thoroughly by inverting the tubes 4-6 times and incubated for 5 min at 25 ^{o}C. The mixture was neutralised using 300 ml of chilled buffer P3, mixed gently by inverting the tubes 4-6 times and incubated on ice for 5 min at room temperature. The suspension was centrifuged (13,226 x g, 30 min, 25 ^{o}C) followed by transfer of the supernatant fraction into a clean microcentrifuge tube.

Meanwhile, Qiagen-tip 20 column was equilibrated by applying 1 ml buffer QBT and allowed to empty by gravitational flow. The supernatant fraction was applied on the column and allowed to enter the resin by gravity. The column was washed four times with 1 ml buffer QC and eluted with 800 µl buffer QF. The DNA was precipitated using 700 µl of isopropanol. The solution was mixed thoroughly and immediately centrifuged (13,226 x g, 30 min, 25 ^{o}C). The DNA pellet was washed with 75% ethanol and re-centrifuged (4,402 x g, 5 min, 25 ^{o}C). The pellet was air-dried and re-dissolved in 35 µl of sterile distilled water. Plasmid concentration was determined using a GeneQuant *pro* Spectrophotometer (Amersham Biosciences).

A.9 Storage of plasmids

Transformed cell containing identified inserts were stored as glycerol stocks as described by Sambrook *et al.*, (1989). To 500 µl of overnight culture, 200 µl of sterile 90 % glycerol was added. The mixture was briefly vortexed and stored at – 70 ^{o}C. To recover the plasmid, the surface of the frozen culture was scraped using a sterile pipette tip and dipped into 5 ml of LB broth and incubated overnight at 37 ^{o}C with moderate shaking (200 rpm). The plasmid was then purified using the method described in A.8.

A.10 Sequencing double-stranded DNA

Double-stranded DNA was sequenced using the ABI PRISM ® BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Optimisation of template concentration ranging from 200 ng to 500 ng and primer concentration between 3.2 to 3.4 pmol was carried out. An optimised template concentration of 500 ng with a primer concentration of 3.3 pmol resulted in good sequences. A 20 µl reaction mixture consisting of 4 µl of BigDye® Terminator v1.1 5X sequencing buffer (ABI PRISM® cat. no. 0405939), 4 µl of BigDye® Terminator v3.1 cycle Sequencing RR-100 (ABI PRISM® cat. no. 0405025), 3.3 pmol of either forward or reverse primers, 500 ng of the plasmid was prepared. The mixture was centrifuged briefly and cycle sequenced using in thermocycler with reaction runs of 25 cycles as 96 ^{o}C for 30 s, 50 ^{o}C for 30 sec and 60 ^{o}C for 4 min.

The unincorporated dye terminators in the sample were removed using DyeEx spin columns packed with prehydrated gel-filtration resin (Qiagen, cat. no. 63106) following manufacturer's instructions and vacuum dried. Prior to sequencing, 18 µl of template suppression reagent (TSR: ABI PRISM® cat. no. 0403142) was added to the dried sample and incubated at 95 ^{o}C for 2 min. The reaction was terminated on ice for 2 min and mixed briefly. The samples were loaded onto an ABI PRISM® 310 genetic analyser, (PE Applied Biosystems, Perkin Elmer).

Alternatively, purified plasmids were sent to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa for sequencing. Sequencing reactions were carried out using the BigDye Version 3.1 terminator cycle sequencing kit (PE Applied Biosystems, Perkin Elmer) and the samples loaded into a Spectrumedix SCE2410 genetic analyser (SpectruMedix LLC, Pennsylvania, USA).

A.11 *Anopheles funestus* cDNA library construction

Construction of the *An. funestus* cDNA library was based on the SMART^{TM} cDNA library construction kit user manual (BD Biosciences, cat no. K 1051-1).

A.11.1 First-strand cDNA synthesis

Poly (A)$^{+}$ mRNA (1 µg) was reverse transcribed to single-strand cDNA with 10 µM SMART IV^{TM} Oligonucleotide (10 µM/µl: 5'-AAG CAG TGG TAT CAA CGA AGA GTG GCC ATT ACG GCC GGC-3') and 10 µM CDS III/3' PCR primer (10 µM/µl: 5'-ATT CTA GAG GCC GAG GCG GCC GAC ATG-

$d(T)_{30}N\text{-}_1N\text{-}3'$) in 0.2 ml microcentrifuge tube. As a control, conventional cDNA synthesis was carried out from 1 μg of human placenta poly $(A)^+$ RNA provided in the kit (1 μg/1 μl). The contents were mixed and the microcentrifuge tube contents spun briefly and incubated at 72 ^oC for 2 min. The contents were cooled on ice for 2 min and spun briefly to collect the contents at the bottom. The volume was adjusted to 10 μl by adding 2 μl of 5X First-Strand buffer (250 mM Tris, pH 8.3, 30 mM $MgCl_2$, 375 mM KCl), 20 mM DDT (20 mM/μl), 10 mM dNTP mix (10mM/μl) and 1 μl PowerScript™ reverse transcriptase. Reverse transcription and template switching was carried out at 42 ^oC for 1 h. Prior to incubation, the contents were mixed by gently pipetting, briefly spun. On completion, the microcentrifuge tube was then placed on ice to terminate first-strand synthesis.

A.11.2 Second strand cDNA synthesis by Long Distance (LD)-PCR

Second strand cDNA was generated from first-strand cDNA by long-distance PCR (Barnes, 1994) for generating full-length cDNA. For LD-PCR, the same temperature (68 ^oC) is applied during annealing and extension with the extension time delayed to obtain integrated cDNA. Into a 0.2 ml microcentrifuge tube, 2 μl of first-strand cDNA was combined with 10 μl of 10X Advantage 2 PCR buffer (400 mM Tricine-KOH, pH 9.2, 150 mM KOAc, 35 mM $Mg(Oac)_2$, 37.5 μg/ml BSA), 20 mM 50X dNTP mix (10 mM/μl), 20 μM 5' PCR primer (10 μM/μl: 5'-AAG CAG TGG TAT CAA CGC AGA GT-3'), 20 μM CDS III/3' PCR primer, 2 μl of 50X Advantage 2 Polymerase mix and 80 μl deionised water. The contents were mixed by gently flicking and centrifuged briefly to precipitate contents at the

bottom of the tube. The mix was placed in a thermocycler and cycled using the following program: 95 ^{o}C for 1 min, 18 cycles of 95 ^{o}C for 5 sec, 68 ^{o}C for 6 min. After completion, 5 µl of the *An. funestus* cDNA and control cDNA synthesized were analysed alongside 1 Kb DNA size marker (Invitrogen, cat. no. 10787-018) on a 1% (w/v) agarose stained using EtBr (10 mg/ml).

A.11.3 cDNA directional cloning

The λTriplEx2 vector facilitates directional cloning with every cDNA inserted into the multiple cloning site (MCS) expressed in all the three reading frames. Following double-stranded cDNA synthesis, proteinase K treatment, *Sfi*1 digestion, chroma-spin purification and ligation in the vector were carried out as described below.

A.11.3.1 Proteinase K digestion

Proteinase K treatment is necessary to inactivate the DNA polymerase activity. Fifty µl of the amplified double stranded cDNA (2-3 µg) was pipetted in a sterile 0.5 ml microcentrifuge tube and 20 µg of proteinase K (20 µg/µl) added. The tube contents were mixed, centrifuged briefly and incubated at 45 ^{o}C for 20 min. After incubation, the contents were again centrifuged briefly. Deionised water (1 vol) and 2 vol of phenol: chloroform: isoamyl alcohol (25:24:1) were added and mixed by continuous gentle inversion for 2 min. To separate the phases, the contents were centrifuged at 13,226 x g for 5 min and the top (aqueous) layer pipetted out

into a clean 0.5 ml microcentrifuge tube. The interface and lower layers were discarded. To wash off the remaining phenol and separate the phases, 2 vol of chloroform: isoamyl alcohol (24:1) was added to the aqueous layer, mixed by continuous gentle inversion for 2 min and centrifuged at 13,226 x g for 5 min. The top (aqueous) layer was pipetted out to a clean 0.5 ml microcentrifuge tube and the interface and lower layers discarded. To the aqueous layer, 1/10 vol of 3 M NaAc (pH 4.2), 26 µg of glycogen (20 µg/µl) and 5.2 vol of room temperature 95% ethanol was added followed by centrifugation (13,226 x g, 20 min, 25 ^{o}C). The supernatant fraction was carefully removed using a pipette without disturbing the pellet. The pellet was washed with 2 vol of 80% ethanol, air-dried to evaporate off residual ethanol. The pellet was re-suspended in 79 µl of deionised water.

A.11.3.2 *Sfi* I digestion and cDNA size fractionation by CHROMA SPIN-400 DEPC

For optimal cloning efficiency, the insert cDNA was phosphorylated. Briefly, 10 µl of 10X Sfi I buffer, 200 units of Sfi I enzyme (20 units/µl) and 1 µl of 100X BSA were added to the re-suspended cDNA (79 µl), in a clean 0.5 ml microcentrifuge tube. The mixture was incubated at 50 ^{o}C for 2 h. Two µl of 1% xylene cyanol dye was added and mixed well. After phosphorylated, the cDNA sample was size-fractionated prior to ligation with the vector. This step was accomplished by gel filtration using CHROMA SPIN-400 DEPC (BD Biosciences, cat no. K 1333) according to manufacturer's instructions. Sample fractions (3 µl) eluted from the column were analysed alongside 1 kb DNA size

marker (Invitrogen, cat. no. 10787-018) on a 1.1% (w/v) agarose gel stained using EtBr (10 mg/ml). Low-molecular weight cDNA fragments less than 400 bp were excluded to eliminate very small inserts in the library. Elutes from the fourth to the sixth well that contained cDNA were pooled to a total volume of 140 µl and stored at –20 ^{o}C.

A.11.3.3 Ligation of cDNA to λTriplEx2 vector

The optimal ratio of DNA to vector in ligation reactions is a critical factor in determining transformation efficiency. In order to obtain the best possible library from the cDNA, a set of three parallel ligations using three different ratios of cDNA to vector was carried out. Ligations of the fractionated cDNA were set up in a total volume of 5 µl. The quantities in each tube consisted of 0.5, 1.0 and 1.5 µl cDNAs. Other components in each tube consisted of 500 ng of the vector (500ng/µl), 0.5 µl of 10 X ligation buffer (500 mM Tris HCl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 0.5 mg/ml BSA), 5 mM ATP (10 mM/µl), 200 units of T4 DNA ligase (400 units/µl) and deionised water. The ligations were incubated at 16°C overnight.

To increase the stability of the library, lambda-phage packaging reaction was performed for each of the ligations using Packagene® Lambda DNA packaging system (Promega, cat. no K3154) according to manufacturer's instructions. The unamplified library was initially titrated followed by amplification of the cDNA library as described in the user manual. The transformation efficiency ratio

between white and blue plaques was determined in the presence of 100 mM IPTG and 100 mM Xgal and calculated as a percentage. Above 80% transformation efficiency was considered a successful ligation. Phage titer was calculated as plaque forming units (pfu)/ml using the formula:

$$\text{pfu/ml} = \frac{\text{number of plaques x dilution factor x } 10^3 \text{ µl/ml}}{\text{µl of diluted phage plated}}$$

During titration of the three test ligations of the library, a phage titer greater than 1 –2 x 10^6 was considered optimal. After packaging, a 1 x 10^6 independent clones in a library was considered optimal. The amplified library was stored in 7% dimethylsulphoxide (DMSO) (v/v) in aliquots of 200 µl at -70 ^oC.

A.11.3.4 Plating of cDNA library

From the calculation of the phage titer (pfu/ml) of the amplified cDNA library (4.9 x 10^8), a dilution of the library with approximately 500 plaques per 90–mm plate was carried out. This was important in maintaining individual plaques during screening. A single, isolated colony from the working stock plate of *E. coli* XL1-blue was picked and used to inoculate 15 ml of LB/maltose/$MgSO_4$ broth (10 g/l Bacto tryptose, 5 g/l Bacto yeast, 5 g/l NaCl, 15 g/l Bacto Agar pH 7.0, 0.2% maltose, 10 mM $MgSO_4$) in a 250 ml flask. The bacterial cells were incubated at 37 ^oC overnight with shaking (200 rpm) until the OD_{600} of the culture reached 2.0. The cells were centrifuged (1957 x g, 5 min), the supernatant fraction poured off and the pellet re-suspended in 7.5 ml of 10 mM $MgSO_4$.

Freshly prepared LB/MgSO4 (10 g/l Bacto tryptose, 5 g/l Bacto yeast, 5 g/l NaCl, 15 g/l Bacto Agar, pH 7.0, 10 mM $MgSO_4$) agar plates (150-mm) were pre-warmed at 37 ^oC for 30 min. The amplified library was diluted in 1X lambda dilution buffer (10X lambda dilution buffer: 1 M NaCl, 0.1 M MgSO4.7H_2O, 0.35 M Tris-HCl, pH 7.5). Dilutions of 1:2, 1:5 and 1:10 were carried out on the amplified library. Twenty µl of each diluent was added to 100 µl of the 1X lambda dilution buffer and 200 µl of XL1-blue overnight culture. The phage was allowed to adsorb at 37 ^oC for 15 min. Two ml of molten top agarose was added to the suspension, mixed by inverting quickly and poured onto the pre-warmed LB/$MgSO_4$ agar plates. The top agarose was allowed to harden and the plates incubated inverted at 37 ^oC overnight or until the plaques were pinpoint in size.

A.12 Screening of the cDNA library for full-length P450 clones

Screening and positive identification of plaques were carried out according to Sambrook *et al.,* (1989) with modifications.

A.12.1 Identification of positive phages

Phage plaques were transferred onto BioBond^{TM} - plus nylon membrane (Sigma, cat. no. N-8656) by plaque lifting procedure. All plaque lifts were performed in duplicate to improve the accuracy of the hybridisation analysis. The plates were pre-chilled to 4 ^oC for at least 1 hr prior to plaque lifts. For each plate, two membrane lifts were prepared. The first membrane was overlaid face down on cold plates bearing bacteriophage plaques without air bubbles between the

membrane and left for 1 min to allow the transfer of phage particles onto the membranes. During the transfer period, the orientation of the membrane to the plate was recorded by stabbing an 18-gauge needle through the membrane into the agar at several asymmetrical points around the edge of the plate. A second membrane was placed onto the plate for 2 min. Each membrane was removed slowly from the plate using a blunt flat forceps and transferred into denaturing buffer (1.5 M NaCl, 0.5 N NaOH) in a 150-mm petri dish for 5 min. The plaques were neutralized for 5 min in neutralization buffer (1.5M NaCl, 0.5M Tris-HCl, pH 7.4). The membranes were shortly rinsed in 2X SSC wash solution (20X SSC: 3 M NaCl, 0.3 M Na citrate, pH 7.0) and placed DNA side up on Whatman paper to air dry. Finally, the DNA was fixed onto the membrane by UV cross-linking in a UV Stratalinker (Stratagene, La Jolla, CA) at 1200 μJ for 1 min.

A.12.2 Preparation of Digoxigenin (DIG)-labelled probe by PCR

Labelling of probes was carried out using PCR probe synthesis kit (Roche Diagnostics, cat. no. 1 636 090) according to manufacturer's instructions. PCR templates were plasmids confirmed as CYP4, CYP6 and CYP9 after sequencing as described in Chapter 2. The PCR mixture consisted of 50 ng plasmid, 5 μM each of SP6 and T7 primers, 2.5 μl of 10X PCR buffer with $MgCl_2$, 2.5 μl of 10X PCR DNA probe synthesis mix, 2 mM dNTP mix and 1.75 units of Enzyme mix Expanded high fidelity (3.5 units/μl) as recommended by the manufacturer. Cycling conditions were as follows: 95 ^{o}C for 2 min, followed by 10 cycles of 95

^{o}C for 30 s, 60 ^{o}C for 30 s, 70 ^{o}C for 40 s, a further 30 cycles of 95 ^{o}C for 30 s, 60 ^{o}C for 30 s, 72 ^{o}C for 40 s was performed and a final extension of 72 ^{o}C for 7 min.

A.12.3 Hybridisation

Plasmids were used as positive controls and confirmed by dot blot hybridisation. Plasmid (50 ng) was blotted and fixed onto BioBond^{TM}- plus nylon membrane (Sigma, cat. no. N-5031). All hybridisations were performed with gentle agitation using DIG Easy Hyb buffer (Roche, cat. no. 1 603 558). All procedures were carried out as described by the manufacturer. Each membrane was equilibrated in 2X SSC buffer for 2 h to remove any agar debri that remained on the membrane and avoid false positives.

The membranes were prehybridised in DIG Easy Hyb buffer (Roche, cat. no. 1 603 558) at 42 ^{o}C for 3 h. Cocktail probes comprising of multiple CYP4, CYP6 and CYP9 genes were denatured at 99 ^{o}C for 5 min and immediately placed on ice. For every 1 ml of pre-hybridisation buffer, 2 µl of the denatured probe was added. For low probe concentrations, upto 4 µl of probe was added to 1 ml of the buffer. The membranes were hybridised overnight at 42 ^{o}C. The hybridisation buffer containing probe was poured into 50 ml Falcon tube and stored at –20 ^{o}C. This buffer was denatured at 68 ^{o}C and re-used in blotting of other membranes. Post hybridisation, the membranes were washed twice at low stringency in 2X SSC, 0.1% sodium dodecylsulphate (SDS) for 5 min at room temperature. The

membranes were washed twice at high stringency in 1X SSC, 0.1% SDS at 65 ^{o}C for 15 min.

The procedure for the immunological detection of DIG-labelled nucleic acids and preparation of working solutions was carried out using the DIG luminescent detection kit (Roche, cat. no. 1 363 514) and the DIG wash and block buffer sets (Roche, cat. no. 1 585 762) from Roche according to manufacturer's protocols. The incubations were all carried out at 25 ^{o}C. The membranes were transferred in a clean container containing 1X wash buffer and washed for 2 min. The membranes were blocked in 1X blocking buffer for 3 h. The DIG-labelled probe was bound onto the membrane for 30 min using antibody solution containing Anti-Digoxigenin-AP diluted at a ratio 1:10000 in 1X blocking solution. The membranes were washed twice for 15 min in 1X washing buffer.

Chemiluminescent detection was carried out using CSPD diluted in 1X detection buffer at a ratio of 1: 500. The membranes were initially equilibrated for 5 min in 1X detection buffer. Each membrane was placed onto cling film containing several drops of CSPD and incubated for 5 min. Excess CSPD was drained off and each membrane transferred onto a dry cling film and sealed. To enhance detection, the membranes were incubated at 37 ^{o}C for 10 min. Each membrane was mounted with the plaque side up onto exposure cassette and a Kodak® Bio Max light film, Light-1 (Kodak, cat. no. Z373494-50EA) placed on top. Films were developed after 30 min exposure.

A.12.4 Positive plaque identification

Replicate membranes were overlaid on a light box and dots that appeared in both replicates in the same orientation were identified as plaques of interest. On a light box, the exposed film with a positive dot was orientated with its corresponding 150 mm plate. The plaque was cored out using 1 ml pipette tip which had a portion of its tip snipped and transferred in 1.5 ml microcentrifuge tube containing 300 ml of 1X lambda dilution buffer and 0.5 μl of chloroform. To release the phage, the solution was incubated at 4 ^{o}C overnight. A second phage library was plated from the phage lysate. The plaques in the secondary plate were hybridised with the same probe as described in section A.11.3. This process was repeated in a tertiary screening or until a desired single plaque was obtained.

A.12.5 Conversion of λTriplEx2 to pTriplEx2

Colonies which turned out to be positive after a tertiary screening were picked with a micro-tip. Conversion to pTriplEx2 *in E.coli* BM 25.8 cells was by *in vivo* excision and circularisation into a complete plasmid from the recombinant phage. This was carried out as described in the SMART^{TM} cDNA library construction kit user manual (BD Biosciences, cat no. K 1051-1). Insert screening was carried out as described in A.7 using 5' (5'-CTC GGG AAG CGC GCC ATT GTT GGT-3') AND 3' (ATA CGA CTC ACT ATA GGG CGA ATT GGC C-3') λTriplEx2 LD-insert screening amplimers except for the cycling conditions, which were carried out as described in the user manual for fragments less than 5 kb. Briefly,

the cycling conditions consisted of 94 ^{o}C for 1 min followed by 30 cycles of 94 ^{o}C for 30 s, 68 ^{o}C for 3 min and a final extension of 68 ^{o}C for 3 min. Sequencing was carried out using 20 μM 5' (5'-TCC GAG ATC TGG ACG AGC-3') and 20 μM 3' (5'-TAA TAC GAC TCA CTA TAG GG-3') pTriplEx2 sequencing primers provided with the kit. Sequence analysis was performed as described in Chapter 2.

A.13 Expression of P450 genes in *An. funestus*

A.13.1 Deoxyribonucleic (DNAse) treatment of total RNA

The quality of RNA is a significant factor in any results obtained in expression studies. To ensure high quality RNA in both blot analysis as well as in quantitative PCR, RNA extractions were carried out as described in Chapter 2 with slight modifications. RNA was extracted from groups of 3 adult mosquitoes. Briefly, total RNA pellet was re-suspended in 20 μl of DEPC-treated water. Total RNA was deoxyribonuclease (DNAse)-treated by adding 1 μl DNAse 1, 19 μl DNAse buffer, 60 μl of DEPC-treated water and incubating at 37 ^{o}C for 30 min. RNA extraction was repeated on the treated sample and the pellet re-suspended in 20 μl of DEPC-treated water. The RNA was quantified using a GeneQuant *pro* Spectrophotometer (Amersham Biosciences) and stored in aliquots (5 μl) at –70 ^{o}C

A.13.2 Dot blot analysis

Dot blot analysis was carried out with a modified protocol from Sambrook *et al*., (1989). Concentrations ranging between 100 to 10 μg of total RNA from FUMOZ-R and FANG were prepared. Serially dilution of each concentration was carried out in 5 μl of DEPC-treated water in 0.5 ml microcentrifuge tubes followed by addition of 10 μl of denaturing solution (2 ml denaturing solution: 1 ml formamide, 356 μl formaldehyde, 200 μl E buffer (10X E buffer: 200 mM MOPS, 50 mM NaAc, 10 mM EDTA), 444 μl DEPC-treated water). The sample was denatured at 65 ^{o}C for 15 min and rapidly cooled in ice. Fifteen μl of ice-cold 20X SSC buffer was added followed by spotting 2 μl onto BioBond^{TM}- plus nylon membrane (Sigma, cat. no. N-5031). The blots were air-dried for 10 min and UV-cross-linked.

Prehybridisation, hybridisation and detection steps were carried out as explained in A.12.3. A cocktail probe each consisting of multiple CYP4, CYP6 or CYP9 cDNAs was used to probe the RNA blots. A cDNA encoding for the ribosomal protein S7 (rsp7) was cloned from *An. funestus* and sequenced (Salazar *et al*., 1993). The plasmid containing the partial fragment of rsp7 was DIG labelled and used as an internal standard in RNA loading in dot blot and northern analysis. The membranes were stripped and rehybridised using rsp7 probe.

A.13.3 Northern Blot analysis

The over-expression of CYP6P9 was validated using northern blot analysis. CYP6P9 probe was used to probe RNA on the membrane. RNA concentrations of FUMOZ-R and FANG ranging from 60 to 5 μg were loaded and separated by denaturing electrophoresis into a 1.2% (w/v) agarose formaldehyde gel (Sambrook *et al*., 1989). Loading concentrations were monitored both by comparing the intensity of the rRNA band on the agarose gel under UV and by probing the same membrane with rsp7 control gene. After electrophoresis, the RNA was transferred onto BioBond^{TM}- plus nylon membrane (Sigma, cat. no. N-5031) by capillary blotting for 16 h using DEPC-treated 20X SSC as transfer buffer. The membrane was washed in 2X SSC, air-dried for 30 min and UV crosslinked. Hybridisation and detection procedures were similar to dot blots. RNA analysis was repeated three times with independent RNA preparations.

APPENDIX B

P450 CLONES

Table B1: CYP4 clones identified as P450 after sequencing.

Clone	***An. gambiae* P450 name**	***An. funestus* P450 name**
CYP4-1	CYP4J5	CYP4J11
CYP4-2	CYP4C36	CYP4C40
CYP4-3	CYP4D22	CYP4D25
CYP4-4	CYP4C35	CYP4C40
CYP4-5	CYP4D15	CYP4D26
CYP4-7	CYP4J5	CYP4J11
CYP4-8	CYP4G17	CYP4G21
CYP4-9	CYP4C27	CYP4C41
CYP4-11	CYP4D15	CYP4D26
CYP4-12	CYP4J5	CYP4J11
CYP4-13	CYP4C25	CYP4C25
CYP4-16	CYP4D22	CYP4D25
CYP4-21	CYP4C35	CYP4C40
CYP4-22	CYP4J5	CYP4J11
CYP4-23	CYP4C35	CYP4C40
CYP4-24	CYP4J9	CYP4J9
CYP4-41	CYP4D17	CYP4D27
CYP4-42	CYP4J10	CYP4J12
CYP4-44	CYP4C35	CYP4C40
CYP4-57	CYP4D17	CYP4D27
CYP4-67	CYP4J10	CYP4J12
CYP4-88	CYP4J10	CYP4J12
CYP4-99	CYP4G17	CYP4G21

Table B2: CYP6 clones identified as P450 after sequencing.

Clone	***An. gambiae* P450 name**	***An. funestus* P450 name**
CYP6-1	CYP6M2	CYP6M8
CYP6-2	CYP6P3	CYP6P9
CYP6-3	CYP6P3	CYP6P9
CYP6-4	CYP6M3	CYP6M7
CYP6-5	CYP6P3	CYP6P9
CYP6-6	CYP6P1	CYP6P1
CYP6-46	CYP6AA1	CYP6AA4

Table B3: CYP9 clones identified as P450 after sequencing

Clone	***An. gambiae* P450 name**	***An. funestus* P450 name**
CYP9-2	CYP9M2	CYP9M3
CYP9-4	CYP9J3	CYP9J12
CYP9-6	CYP9J5	CYP9J11
CYP9-8	CYP9J5	CYP9J14
CYP9-9	CYP9J5	CYP9J11
CYP9-10	CYP9M2	CYP9M3
CYP9-11	CYP9J5	CYP9J11
CYP9-12	CYP9J5	CYP9J11
CYP9-13	CYP9J3	CYP9J12
CYP9-14	CYP9M2	CYP9M3
CYP9-15	CYP9L2	CYP9L2
CYP9-16	CYP 9J5	CYP9J11
CYP9-19	CYP 9M2	CYP9M3
CYP9-23	CYP 9J3	CYP9J12
CYP9-27	CYP 9J3	CYP9J12
CYP9-28	CYP 9M2	CYP9M3
CYP9-30	CYP9J5	CYP9J11
CYP9-31	CYP9J5	CYP9J11
CYP9-32	CYP9J5	CYP9J11
CYP9-35	CYP9J4	CYP9J13
CYP9-36	CYP9J5	CYP9J11
CYP9-37	CYP9J5	CYP9J11
CYP9-38	CYP9J4	CYP9J13
CYP9-39	CYP9J3	CYP9J12
CYP9-40	CYP9M2	CYP9M3
CYP9-43	CYP9J5	CYP9J11
CYP9-44	CYP9J5	CYP9J11
CYP9-45	CYP9J4	CYP9J13
CYP9-46	CYP9J4	CYP9J13
CYP9-47	CYP9J5	CYP9J11
CYP9-48	CYP9M2	CYP9M3
CYP9-56	CYP9M2	CYP9M3

APPENDIX C

FLUORESCENCE ACQUISITION GRAPHS

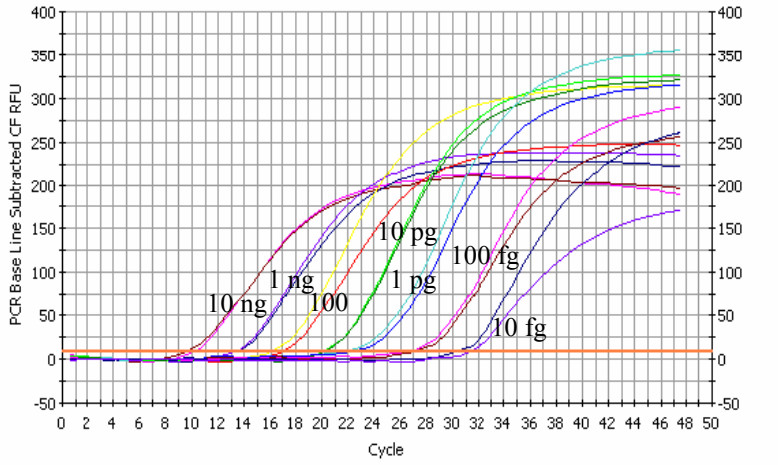

Fig. C1: **SYBR Green fluorescence acquisition by PCR product from**

serially diluted CYP6P9 standard plasmid.

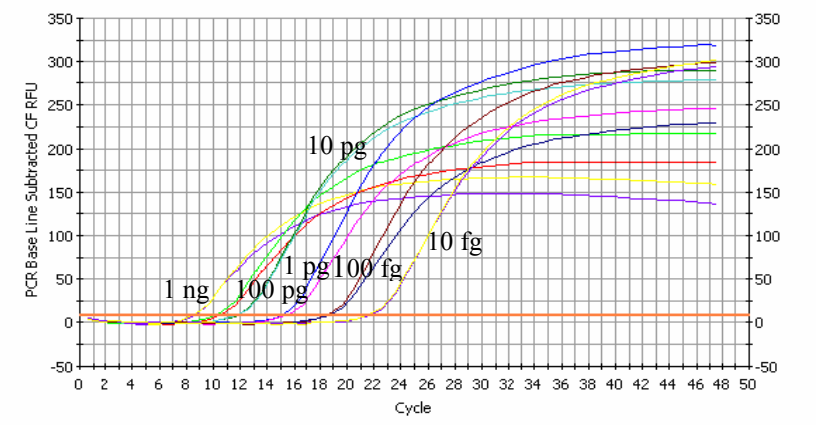

Fig. C2: **SYBR green fluorescence acquisition by PCR product from serially diluted rsp7 standard plasmid.**

APPENDIX D

PUBLICATION

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