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

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CHAPTER 1

Literature review

1.1 Malaria in Africa

Malaria is a common and life-threatening disease in many tropical and subtropical developing countries. There were an estimated 881 000 malaria deaths during 2006, of which 91% were in Africa and 85% were of children under 5 years of age (WHO, 2008). Malaria is responsible for one out of every four childhood deaths in Africa and the number of clinical cases of malaria each year outweighs the number of cases of AIDS and Tuberculosis combined. Malaria is distinctly seasonal in South Africa with the highest risk being during the wet summer months (September to May) (Department of Health, 1995).

Malaria is caused by parasites of the species *Plasmodium* that are spread from person to person through the bites of infected female *Anopheles* mosquitoes. There are five types of human malaria - *Plasmodium falciparum*, *P. vivax, P. malariae*, *P. ovale* and more recently *P. knowlesi* (WHO, 2010). *Plasmodium falciparum* and *P. vivax* are the most common, however only *P. falciparum* is associated with severe morbidity and mortality (WHO, 2006). Daneshvar *et al*. (2009) confirmed that knowlesi malaria is a significant cause of morbidity in the Kapit Division (Sarawak, Malaysia) and approximately 1 in 10 patients develop potentially fatal complications.

Mosquitoes belong to the Class Insecta, Order Diptera and Family Culicidae. There are 34 genera that belong to three Subfamilies, namely Anophelinae, Toxorhynchitinae and Culicinae. The genus *Anopheles* contains vectors of malaria parasites as well as arboviruses

(Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; AMCA, 2010) while the Culicinae (which includes the genera *Aedes*, *Culex*, and *Mansonia)*, contain the mosquito vectors for filariasis and various arboviruses (White, 2002). The bulk of malaria transmission in Africa is caused by three major vectors, *An. gambiae s.s, An. arabiensis* and *An. funestus s.s* (Gillies and De Meillon, 1968; White, 1974; Gillies and Coetzee, 1987). *Anopheles gambiae s.s* and *An. arabiensis* are members of the *An. gambiae* complex while *An. funestus s.s* is a member of the *An. funestus* group. Being able to identify and distinguish mosquito species is important for planning disease surveillance and implementing effective control measures. This study focuses on *An. funestus* and the other species will therefore not be discussed in detail here.

1.1.1 The Anopheles funestus group

Anopheles funestus Giles historically belonged to a group of nine African species (*An. funestus s.s., An. rivulorum* Leeson, *An. leesoni* Evans, *An. vaneedeni* Gillies & Coetzee, *An. parensis* Gillies, *An. confusus* Evans & Leeson, *An. aruni* Sobti, *An. fuscivenosus* Leeson, and *An. brucei* Service) (Gillies and de Meillon, 1968; Gillies and Coetzee, 1987). All these species are morphologically similar and makes it difficult to identify individual members of the group using adult characteristics, although immature features can distinguish some of the species (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Since then two additional species belonging to the *An. funestus* group have been reported. *Anopheles rivulorum-like* was described from Cameroon by Cohuet *et al.* (2003). The species-specific PCR assay (Koekemoer *et al.*, 2002) was supplemented by a primer specific to *An. rivulorum*-like, which annealed in a region of the ITS2 sequence where 8 of 21 nucleotides allowed distinction between both *An. rivulorum*; ensuring its specificity (Cohuet *et al*., 2003). More recently a new species of the *An. funestus* subgroup has been identified from Malawi via molecular,

cytogenetic, and cross-mating studies (Spillings *et al*., 2009). This new species has been provisionally named *An. funestus*-like until a formal description is published (Spillings *et al.*, 2009). It occurs in sympatry with *An. funestus* and as it associates with human habitations, further investigations and collections are needed to determine this species vector status.

The species-specific multiplex polymerase chain reaction (PCR) assays (Koekemoer *et al.*, 2002; Spillings *et al*., 2009) as well as cytogenetics (Green and Hunt, 1980) helps to distinguish these species from one another and is useful for identifying the five members of the *Anopheles funestus* group. Cytogenetics is more accurate than morphological examination however it can only be used to identify half-gravid females of *An. parensis*, *An. rivulorum*, *An. leesoni*, *An. fuscivenosus* and *An. confusus* (Green, 1982). Evidence of genetic heterogeneities (the situation in which different mutant genes produce the same phenotype) within *An. funestus* was revealed during early cytogenetic investigations (Green and Hunt, 1980). Polymorphic inversions are found in several populations in East (Kamau *et al.*, 2002), South (Green and Hunt, 1980; Boccolini *et al*., 2005), Central (Cohuet *et al*., 2005; Dia *et al*., 2000a) and West Africa (Lochouarn *et al*., 1998; Constantini *et al*., 1999; Dia *et al.*, 2000b). More recent cytogenetic studies in west Africa has shown clear evidence of genetic differentiation in sympatric populations of *An. funestus*, indicating that this taxon may consist of a complex of cryptic species (Lochouarn *et al*., 1998; Constantini *et al*., 1999). Cytogenetic analysis of the polytene chromosomal banding patterns of the Malawian specimens displayed homosequential banding arrangements with *An. funestus*, but were fixed for the inverted arrangements 3a, 3b, and 5a, which are commonly polymorphic in *An. funestus* (Spillings *et al.*, 2009).

Anopheles funestus s.s is one of the primary malaria vectors in sub-Saharan Africa due to its highly anthropophilic (biting man) and endophilic (resting indoors) behaviours, making it especially vulnerable to control by indoor residual spraying (IRS) assuming effectiveness of the insecticide employed (Gillies and De Meillon, 1968).

1.1.2 Malaria vector control and malaria prevention

Malaria prevention includes measures taken both against mosquito vectors and against the malaria parasite. Parasite control aims to significantly reduce both the number and rate of parasite infections and clinical malaria cases. Vector control is specifically aimed at controlling the mosquito population and thereby reducing and/or interrupting transmission (WHO, 2010). The combination of tools and methods to combat malaria includes long-lasting insecticidal nets (LLIN), indoor residual spraying of insecticides (IRS) and artemisinin-based combination therapy (ACT), supported by intermittent preventative treatment in pregnancy (IPT) (WHO, 2008). By June 2008, all except four countries and territories worldwide had adopted ACT as the first-line treatment for *P. falciparum* (WHO, 2008). The 2005 World Health Assembly (WHA) specified that, as a result of these interventions, malaria cases and deaths per capita should be reduced by $\geq$ 50% between 2000 and 2010, and by $\geq$ 75% between 2005 and 2015 (WHO, 2008). Other measures entail personal protection to avoid mosquito bites and the use of antimalarial drugs (chemoprophylaxis). Despite these interventions, only in Benin, Cameroon, Central African Republic, Gambia, Ghana, Uganda and Zambia were more than 50% of all children treated with an antimalarial drug (WHO, 2008).

The main class of insecticide used by vector control programmes is pyrethroids. However, pyrethroid resistance has increased in the last few years (Santolamazza *et al.,* 2008) and new

pesticide products have not been developed in over 30 years due to lack of investment (Ranson *et al*., 2010). As a result, vector control efforts have been undermined. Currently, promising new insecticide formulations are being evaluated by the Innovative Vector Control Consortium but it will still be many years before any of these alternatives can be implemented (Ranson *et al.*, 2010). For these reasons, effective resistance management strategies are imperative in Africa.

Insecticide resistance management is an integral part of vector control as it is designed to monitor and circumvent or reduce the development of insecticide resistance in affected populations, as well as to prevent the development of resistance in unaffected populations (WHO, 1998). Introduction of inappropriate insecticides without a proper understanding of the prevailing resistance mechanisms may lead to enhanced vector resistance and disease control failure. Early detection and knowledge of the resistance status and the underlying mechanisms in vector mosquitoes are essential for effective long-term control of the vector.

1.2 The different classes of insecticides

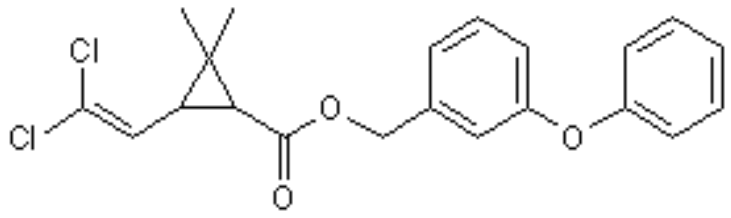
Insecticides are classified according to their chemical structures, and each insecticide has three names: the common name, the trade name, and the chemical name (Yu, 2008). There are four classes of insecticides, namely carbamates (esters of carbamic acid), organophosphates (phosphoric acid derivatives, consisting of six subclasses), organochlorines (also known as chlorinated hydrocarbons) and pyrethroids (consist of pyrethrum and its synthetic pyrethrum analogs called synthetic pyrethroids) (reviewed in Yu, 2008). As this study focuses on pyrethroid resistance, more detail will be given on this class of insecticide only.

1.2.1 Pyrethroids

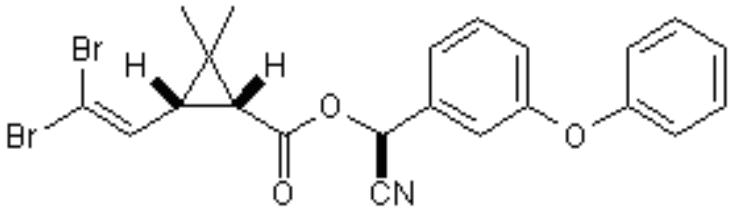
Pyrethroids are synthesized derivatives of naturally occurring pyrethrins, which are taken from pyrethrum, an extract of dried Chrysanthemum flowers (ETN, Pyrethroids, 1994). Pyrethroids are toxic to most insects, both disease vectors as well as beneficial insects, however recent research by Johnson *et al.* (2006) found that honey bees, often thought to be extremely susceptible to insecticides in general, exhibited considerable variation in tolerance to pyrethroid insecticides. As they are naturally unstable, pyrethrins are chemically modified to make them more stable, more efficient and have better residual activity in the open-field conditions (Elliott *et al.*, 1978; Vijverberg and Bercken, 1982; Gray and Soderlund, 1985; Smith and Stratton, 1986; Coats *et al.*, 1989; Haya, 1989; ETN, Pyrethroids, 1994; Konanz, 2009). Pyrethroids are generally fast-acting poisons if ingested or through direct tarsal contact and act by paralyzing the nervous system of insects producing the 'knockdown' effect (Bloomquist, 1993; Dong, 2007).

Pyrethroids target voltage-gated sodium ion channels in nerve axons, whereby they prolong the opening of these channels by altering the gating kinetics, leading to hyperexcitability, bursts of action potentials, nerve blockage and finally death (Bloomquist, 1993; ETN, Pyrethroids, 1994; Tomlin, 2006; Dong, 2007; Konanz, 2009). Pyrethroids are often formulated with oils and packaged in combination with synergists, such as piperonyl butoxide (PBO) (Gosselin *et al.*, 1984). Synergists increase the potency of a pesticide, and PBO acts by inhibiting microsomal oxidase enzymes responsible for the breakdown of toxins (and insecticides) leading to the death of the insect.

There are two classes of pyrethroids, namely Type I (those lacking a cyano group) and Type II (containing an alpha-cyano group) (Gammon *et al.*, 1981; Eisler, 1992). Permethrin is a Type I pyrethroid and deltamethrin is a Type II pyrethroid (Figure 1.1). Permethrin resembles pyrethrins chemically, but it is chlorinated to increase its stability. There are four isomeric forms; two *cis* and two *trans,* which differ in the spatial arrangement of the atoms. The *cis*-isomer has been noted to be more toxic as it is not as easily hydrolysed as the *trans*-isomer (Shono *et al*., 1978; Muller *et al*., 2008). Both Type I and Type II pyrethroids act on the central nervous system of insects and interfere with sodium channels to disrupt the function of neurons causing muscles to spasm, culminating in paralysis and death (ETN, Pyrethroids, 1994; Tomlin, 2006; US-EPA, 2007). Some Type II pyrethroids also affect the action of a neurotransmitter called gamaaminobutyric acid (GABA) (Costa, 1997).



permethrin



deltamethrin

Figure 1.1: Structures of the Type I (permethrin) and Type II (deltamethrin) pyrethroids (adapted from www.inchem.org)

1.3 Mechanisms of resistance

Resistance, is an expansion and fixation of adaptive genomic alterations driven by allelochemical and/or insecticide selection (Li *et al*, 2007) and can also serve as an example of natural selection whereby the insecticide prevents susceptible individuals from reproducing leaving only those individuals carrying the gene for resistance (Pinto *et al.*, 2007). The WHO defines resistance as the "development of an ability in a strain of organism to tolerate doses of toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of species" (cited by Zlotkin, 1999). There are two main groups of resistance mechanisms, namely behavioural resistance and physiological resistance. Reduced penetration, target-site insensitivity and increased detoxification (metabolic resistance) fall into the latter group (Yu, 2008). Cuticular (reduced penetration) and behavioural resistance are less well studied mechanisms (Ranson *et al.*, 2010; Wood *et al*., 2010). These four mechanisms are briefly described below however increased detoxification, which involves the cytochrome P450s, is described in more detail.

1.3.1 Behavioural resistance

This is the least understood mechanism compared with the physiological resistances and has been defined as the development of an ability to avoid a dose of insecticide that would prove lethal (Yu, 2008). The insects simply stop feeding if they come across certain insecticides or leave the area altogether. This mechanism is stimulus dependent and is a matter of hypersensitivity and hyperirritability (Yu, 2008). Such mechanisms have been observed in resistant diamondback moths (Moore *et al*., 1989) and German cockroaches (Silverman and Bieman, 1993).

*1.3.2 **Reduced penetration***

By itself this mechanism provides only slight resistance (Plapp, 1986). Penetration resistance to insecticides has been related to cuticle thickening, whereby resistant strains absorb the toxin more slowly due to more protein and lipid in the cuticle than its susceptible strain, as well as an increase in density and hardness in the cuticle which probably decreases its permeability to insecticide molecules (Vinson and Law, 1971; Ahmad *et al*., 2006; Wood *et al.*, 2010).

*1.3.3 **Target-site insensitivity***

Target-site resistance or knock-down resistance (kdr) involves changes in the sodium ion channel of insects that prolongs their exposure times to pyrethroid insecticides and has been well documented in insects (Bloomquist, 1996; Martinez-Torres *et al*., 1997; Chen *et al.*, 2010). Decreased sensitivity of other major insecticide target sites, such as acetylcholinesterases (AchEs) and gama-aminobutyric acid (GABA)-gated chloride channel, have also been reported (Mc Caffery, 1998; Vais *et al.*, 2001).

The target site where the insecticide acts in the insect may be genetically modified to prevent the insecticide binding or interacting at its site of action, reducing its pesticidal effect. The most common amino acid replacement associated with pyrethroid resistance in malaria vectors is a substitution of the leucine residue found at codon 1014 with either phenylalanine (1014F) or serine (1014S) (Martinez-Torres *et al*., 1998; Ranson *et al*., 2000). Target-site insensitivity is associated with resistance to pyrethroids and DDT although not exclusively (Davies *et al.*, 2007; Balkew *et al*., 2010).

1.3.4 Metabolic-mediated resistance

Throughout an insect's life cycle it is faced with numerous interactions with natural toxins (xenobiotics) from plants (allelochemicals) and synthetic man-made toxins (insecticides) from which the insect must protect itself. Over time insects have biologically adapted to combat these toxins by various mechanisms, such as detoxification, which differ in the level and type of mechanism amongst different stages, populations and species of insects (Yu, 2008), as well as other strategies such as sequestration, scavenging and binding (Sheehan *et al.*, 2001). Knowledge of detoxification helps us to understand mechanisms of insecticide resistance and hence develop sound resistance management (Yu, 2008).

Detoxification enzyme-based resistance occurs when increased activity of esterases, cytochrome P450 monooxygenases and glutathione-S-transferases (GSTs), sequesters or detoxifies the insecticide to impair the toxicity of the insecticide before it reaches its target site (Brogdon and Mc Allister, 1998; Liu *et al*., 2006). The cytochrome P450s are the primary enzyme family responsible for pyrethroid metabolism in insects and metabolize insecticides through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester oxidation, and nitrogen and thioether oxidation (Wilkinson, 1976; Brogdon and Mc Allister, 1998).

It has been well documented that P450-mediated pyrethroid resistant insects have higher levels or more efficient enzyme forms of one or more P450s compared to susceptible insects and that resistant insects can be rendered susceptible using synergists such as piperonyl butoxide (Feyereisen, 1999; Brooke *et al.*, 2001). Detoxifying enzymes are, and always have been, required by organisms to cope with ongoing exposure to toxins in the environment and it is

also not unusual to find high homology in P450 genes between mosquito species that share a common ancestor (Ranson *et al*., 2002; Amenya *et al.*, 2005). It has been shown in *Culex pipiens* (Baldridge and Feyereisen, 1989) and *Aedes aegypti* (Sanders *et al.*, 2003) that the upregulation of cytochrome P450s occurs in response to a blood meal. Spillings *et al.* (2008) also showed that the presence of a blood meal prior to insecticide exposure, leads to further stimulation of insecticide detoxification mechanisms and a decrease in insecticide susceptibility in the pyrethroid resistant strain of *An. funestus.* Knowledge on this complex resistance mechanism is continuously enhanced through new information. This increased metabolism can result from modifications of existing enzymes making them more suited to degrade the insecticide, or the over-production of these detoxification enzymes, which in susceptible insects, occurs in much lower quantities (Hemingway *et al.*, 1999; Siegfried and Scharf, 2001).

The biochemical mechanisms involved in the transformation of insecticides have been classified into three distinct phases which involve the conversion of lipophilic, non-polar xenobiotics into more water-soluble and less toxic metabolites (phases I and II), that can then be eliminated more easily from the cell (phase III) (Sheehan *et al*., 2001). Phase I mainly involves the catalytic activity of the cytochrome P450 system and is therefore further discussed. Phase I reactions consist of oxidation, hydrolysis and reduction; with oxidation appearing to be the most important and involves the microsomal P450 proteins (Guengerich, 1990). The main function of this phase is to decrease the biological activity of the toxicant using rate-limiting enzymes (with respect to toxicity) so that it can be excreted (if polar enough) but is usually further converted by phase II reactions (Yu, 2008).

1.4 Pyrethroid resistance

Pyrethroid insecticides remain the first choice of chemical for IRS due to their low mammalian toxicity, rapid breakdown in the environment and residual efficacy and are also the only class of chemicals allowed for impregnating bednets (WHO, 2005; cited by Amenya *et al.*, 2005). Pyrethroid resistance in vector mosquitoes has recently been reported throughout Africa, for example in southern Africa (Hargreaves *et al*., 2000), Nigeria (Awolola *et al*., 2002), Cameroon (Chouaibou *et al*., 2008), Cote d'Ivoire (Tia *et al*., 2006) Ghana (Coetzee *et al*., 2006) and Benin (N'Guessan *et al*., 2007). This threatens to compromise vector control interventions (Elissa *et al*., 1993; Vulule *et al*., 1994; Hargreaves *et al*., 2000; Awolola *et al*., 2002; Stump *et al*., 2004).

Resistance is not evolving through unique new mechanisms; rather, existing mechanisms are being enhanced, and cross-resistance (eg. DDT-permethrin) is occurring (Brogdon and Barber, 1990; Miyazuki *et al*., 1996; Williamson *et al.*, 1996).

1.4.1 Pyrethroid resistance in Anopheles funestus from southern Africa

Resistance to pyrethroids is developing at an alarming rate in *Anopheles* mosquitoes in Africa. Pyrethroid resistance in *An. funestus* has spread rapidly in the past few years (Hunt *et al.*, 2010; Cuamba *et al.*, 2010; Morgan *et al.*, 2010; Kloke *et al*., 2011) and has disrupted malaria control in southern Africa (Hargreaves *et al.*, 2000; Casimiro *et al*., 2006). Identification and characterization of the specific genes involved in pyrethroid resistance in resistant *An. funestus* is an important step towards improving current vector control in southern Africa. Initial studies showed that pyrethroid resistance in populations of *An. funestus* southern Mozambique was mediated by increased monooxygenase (P450) insecticide metabolism (Brooke *et al.*,

2001). There are no reports of target site resistance in *An. funestus* to date, and instead, resistance is mediated by the over-expression of P450 enzymes.

1.5 Cytochrome P450 monooxygenases

The cytochrome P450 monooxygenases [also known as mixed-function oxidases (MFO), microsomal oxidases, or P450 enzymes] are a ubiquitous superfamily of enzymes involved in the catabolism and anabolism of xenobiotics (drugs, pesticides and plant toxins) and they are involved in regulating hormones, fatty acids and steroids (Bridges *et al*., 1998; Scott, 1999). They were first discovered in 1955 in rat liver microsomes and they are characterized by an intense Soret absorption peak at 450nm in its reduced form (carbon-monoxide-bound form) (De Smet *et al.,* 1998; Werck-Reichhart and Feyereisen, 2000). Cytochrome P450s (P450s) can be identified by a common sequence in the heme binding region (Scott, 1999; Scott *et al.,* 2001; Scott and Wen, 2001). These P450s are generally located in the endoplasmic reticulum or mitochondrion, and require cytochrome P450 reductase (CPR) for reducing equivalents, and sometimes require cytochrome b5 for enhancing the activity of some P450s (Berge *et al*., 1998; Bridges *et al*., 1998; Werck-Reichhart and Feyereisen, 2000; Scott and Wen, 2001; Feyereisen, 2005). Insect monooxygenases are found in many tissues and are most enzymatically active in areas such as the fat bodies, malphigian tubules and the midgut, but particularly in microsomes (endoplasmic reticulum-bound) (Hodgson, 1983; Feyereisen, 1999; Scott, 1999).

There are two types of P450 systems, namely bacterial/mitochondrial (type I) and microsomal (type II). Microsomal cytochrome P450s are a three-component system comprising cytochrome P450, cytochrome P450 reductase (CPR), and a phospholipids

(phosphatidylcholine) (Paine *et al*., 2005; Yu, 2008). P450s and their associated P450 reductases have broad substrate specificity, genetic diversity, and catalytic versatility, making them the only metabolic system that can mediate resistance to all classes of insecticides (Feyereisen, 2005).

The P450s are classified into thirty-six gene families based on the comparison of deduced amino acid sequences (Nelson, 2005; Zhou and Huang, 2002). Nomenclature for P450s is as follows: sequences are named CYP, followed by a number, a letter and a number indicating the family, subfamily and isoform, respectively (Nelson *et al.*, 1996). In a table compiled by Baldwin *et al.* (2009) which compares the number of functional P450 genes in different genomes, he states that *Drosophila melanogaster* have 83 and mice have 102. Ranson *et al.* (2002) stated that there are 111 putative *Anopheles gambaie s.s* P450s. To date, over 660 insect P450 genes, distributed across 48 P450 families, have been characterized based on amino acid sequence similarities, and include families and subfamilies such as CYP4, CYP6, and CYP9 (Li *et al*., 2007; http://drnelson.utmem.edu/CytochromeP450.html).

The majority of the P450 genes associated with insecticide resistance are from four main families, namely CYP4, 6, 9 and 12 (Feyereisen *et al*., 1989) with the CYP6 genes involved in xenobiotic metabolism as well as insecticide resistance (Feyereisen, 2005; Yu, 2008). Enhanced levels of oxidases in resistant insects result from constitutive over-expression rather than gene amplification (Tomita and Scott, 1995; Carino *et al.*, 1994). Twenty five P450 genes have been reported to be overproduced via upregulation in various insecticide resistant insects (Li *et al.*, 2007). The P450s of family 4 are mainly important in detoxification systems of insects and in lipid metabolism (Wei *et al*., 2000; Estabrook, 2003; Rewitz *et al*., 2006;

Kang *et al.,* 2007; Li *et al*., 2007). In addition, they are also essential for the function of the sensory organs such as antennae in insects. Here, they may be involved in odorant clearance (Maibeche-Coisne *et al.,* 2005). The hormone structure is also dependent on P450s (Rewitz *et al.,* 2006) and therefore small differences in the hormones utilized (i.e. juvenile hormones, methyl farnesoate) may be dependent on these enzymes availability or the timing of expression (Baldwin *et al*., 2009).

1.5.1 Reaction and enzymatic cycle

Microsomal P450s require electrons for catalysis, and these are donated by the electron donors [CPR and cytochrome b5 (b5)] (Yamazaki *et al.,* 1999a). The details of the catalytic events mediated by cytochrome P450 are not fully understood. However, the overall reaction occurs according to the equation: $RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$ where RH is the substrate and ROH is the oxidized product (Guengerich, 1991; Yu, 2008). The enzymatic cycle includes substrate binding, first electron transfer, oxygen binding, second electron transfer, substrate oxidation, and finally product dissociation (Bridges *et al*., 1998). It is CPR which donates the first and second electrons to P450. Due to its redox potential (+ 25 mV), b5 can only donate the second electron to P450 (Vergeres and Waskell., 1995). *Anopheles funestus* CPR was isolated and found to have a 96.5% identity with that of *An. gambiae* CPR (Matambo, 2008; Matambo *et al*., 2010). In theory, without CPR functioning in the cell, or if the CPR gene was silenced, most P450 monooxgenase activity would decrease or disappear completely (Schuler and Werck-Reichhart, 2003).

1.5.2 The CYP6 family

The CYP6 family is found exclusively in insects and is the most extensively studied P450 group in insects (Liu and Scott, 1998; Kasai *et al.,* 1998, Winter *et al.,* 1999; Wen and Scott, 2001; Nikou *et al.,* 2003; Rongnoparut *et al,* 2003; Rodpradit *et al*., 2005). CYP6 genes have been reported to be involved in metabolism of plant defensive chemicals (Scott *et al*., 1998; Kahn and Durst, 2000) and insecticides (Feyereisen, 1999; Scott, 1999; Scott and Wen, 2001). Genes within this family have also been shown to be associated with pyrethroid resistance in *An. funestus* (Wondji *et al.,* 2007; Matambo, 2008; Matambo *et al.,* 2010; Christian *et al*., 2011). Other cases for the involvement of this family in insecticide resistance have also been documented in the house fly (CYP6A1), (Andersen *et al.,* 1994; Feyereisen *et al*., 1989), CYP6D1 (Tomita and Scott, 1995) and CYP6A2 (Brun *et al.,* 1996; Waters *et al.,* 1992).

The genomic changes (in *cis* or *trans)* leading to over-expression, such as those found for CYP6G1 alleles in *D. melanogaster* and its sister species *D. simulans* (Catania *et al.,* 2004; Daborn *et al.*, 2002), have been attributed to i) upregulation via mutations in *trans*-regulatory loci and/or *cis*-acting promoter sequences, ii) upregulation via indels or mutations in *cis*-acting elements, iii) resistance via coding sequence changes, and iv) defining catalytic activity (Li *et al.,* 2007). However, the process of how these genomic changes contribute to over-expression of resistant P450 genes still needs to be resolved.

1.5.3 The multiplicity of P450s

There are multiple known substrates and unique P450 sequences; however their versatility and potential for industrial purposes have generated a great deal of interest in understanding their structure, function and redox reactions (Bridges *et al*., 1998). It has been established that

insect cytochrome P450 exists in multiple isoforms, ranging from two in the black swallowtail, *Papilio polyxenes*, up to as many as six forms in the housefly, *Musca domestica* (Yu, 2008). An isoform is a protein that has the same function as another protein but is encoded by a different but related gene and may have small differences in its sequence (Merriam-Webster.com, isoform: http://www.merriam-webster.com/dictionary/isoform), arising from either single nucleotide polymorphisms, differential splicing of mRNA, or post-transcriptional or post-translational modifications (e.g. sulfation, glycosylation, etc.). This multiplicity would explain their diverse functions, distributions, broad substrate specificity and capabilities for oxidizing various lipophilic compounds (Yu, 2008). The amazing ability of P450s to metabolize a wide variety of compounds makes them the subject of active research in many fields (Scott and Wen, 2001).

P450s also show diversity in their expression related to life stages, sexes, tissues, strains or diet (Scott and Wen, 2001). Expression of individual P450s can be ubiquitous (eg. CYP6D1, reviewed in Korytko and Scott, 1998; Scott and Lee, 1993) or specific (eg. CYP6L1 in male reproductive tissues, reviewed in Wen and Scott, 2001).

By isolating individual P450s, research into the areas of insecticide resistance, plant-insect interactions and insect physiology can be investigated to improve our knowledge. Characterisation of the specific P450s involved in pyrethroid resistance are necessary for the identification of specific markers that could be used to design diagnostic assays to detect and monitor this resistance (Cuamba *et al.,* 2010), as well as being essential for understanding population genetics and evolution of resistance, and for the design of effective counter-measures for resistance.

1.5.4 Measuring P450 activity

The most sensitive and rapid system used to detect monooxygenase activity in insects is the use of 7-ethoxycoumarin to measure fluorometrically monooxygenase activity via O-deethylation (Berge *et al*., 1999). Two of the commonly used assays in this field are the measurements of total P450 and NADPH-P450 reductase in biological preparations (Guengerich *et al.*, 2009). In the P450 spectral assay, the ferrous form of the hemeprotein reacts with the carbon monoxide (CO) to form a complex that specifically produces a spectrum with a wavelength at ~ 450nm, owing to the signature cysteine thiolate axial ligand to the heme iron in these proteins (Guengerich *et al.*, 2009). The extinction coefficients were developed by Omura and Sato (1962; 1964). This assay is useful for measuring total P450 in a biological sample and in the use of recombinant P450 preparations it can measure the stochiometry of active P450 per unit protein (Guengerich *et al*., 2009). As P450s become less active, the conversion to the form of a 420nm peak is observed (Omura and Sato, 1967).

1.6 The focus on CYP6P9 and its duplicated gene CYP6P13 in *An. funestus*

Brooke *et al*. (2001) showed that increased P450 activity is associated with permethrin resistance in *An. funestus* from southern Africa. Additional evidence was obtained when resistant *An. funestus* mosquitoes synergized with PBO resulted in 100% mortality, supporting the role of monooxygenases in pyrethroid resistance. Amenya *et al.* (2005) isolated the first 31 partial P450 gene fragments from *An. funestus* using degenerate primers based on *An. gambiae* P450 sequences. CYP6P9 in *An. funestus* has been identified as being the prime candidate for conferring pyrethroid resistance (Wondji *et al*., 2007; Amenya *et al*., 2008; Wondji *et al*., 2009). Wondji *et al.* (2009) identified four genes, *CYP6P9*, *CYP6P4*,

CYP6AA4, and *CYP6P1*, to be significantly differentially expressed (P<0.001) in *An. funestus* resistant and susceptible strains.

CYP6P9 is a microsomal membrane bound protein containing the highly hydrophobic N-terminal region and the PERF motif that is present in all microsomal cytochrome P450s (Matambo, 2008). *Anopheles funestus* CYP6P9 is the ortholog of *An. gambiae* CYP6P3 and shows a sequence identity of 86% (Matambo *et al*., 2010). *In-situ* hybridization and quantitative trait locus (QTL) mapping by Wondji *et al.* (2007) confirmed that the pyrethroid (permethrin) resistance locus was on chromosome arm 2R, mediated by one or a cluster of P450 enzymes specifically from the CYP6 gene family and accounts for over 60% of the resistance phenotype but also implies that other factors may play a role as well. It has been postulated that up-regulation is mediated via mutations in a promoter or a *cis*-acting regulatory region but so far there has been no evidence supporting this (Amenya *et al*., 2008).

It was further shown that the expression of CYP6P9 in adult mosquitoes is dramatically increased between the ages of three-days old and 14-days old and the effect is more pronounced in females. However, CYP6P9 expression is not sex specific and has been shown to be involved in the metabolism of sex hormones (Amenya *et al.,* 2008).

CYP6P9 has recently been shown to be one of two duplicate genes, the other being CYP6P13 (Wondji *et al*., 2009; Matambo *et al*., 2010) sharing 93.7% sequence identity with CYP6P9 (Matambo *et al*., 2010). CYP6P9 and CYP6P13 have 29 variant amino acids and both have a predicted length of 509 amino acids (Wondji *et al*., 2009). CYP6P9 and CYP6P13 were confirmed to be over-expressed using qPCR analysis in *An. funestus* from Mozambique

(Christian *et al.*, 2011). Extensive gene duplication within the mosquito detoxification gene family may enable up-regulation of various enzymes that alternatively contribute to insecticide resistance (Muller *et al*., 2007). This tandem duplication has been suggested to contribute to the resistance phenotype seen in the FUMOZ-R strain. It has also been suggested that P450 duplication of CYP6P9 and CYP6P4 increases pyrethroid metabolism in resistant *An. funestus* mosquitoes, which may generate genes that are more metabolically active against insecticides (Matambo, 2008; Wondji *et al.*, 2009).

Further to the difference in gene transcription levels, Matambo *et al.* (2010) reported four amino acid differences in the F-G loop and outside the conserved regions between the resistant and susceptible populations (Matambo *et al.*, 2010). This might be significant since the F-G loop, in other P450s, operates as a lid, opening and closing to allow the entry and exit of substrates to the active site (Scott *et al.*, 2003; Poulos, 2003). These variations and structural differences may have an impact on the binding of substrates, affecting the enzymes functionality and involvement in insecticide resistance (Matambo *et al.*, 2010). Differences in amino acid composition between resistant and susceptible strains in *Drosophila melanogaster* CYP6A2 is associated with DDT resistance (Bergé *et al.*, 1998; Amichot *et al.*, 2004).

Apart from this gene's association with resistance and its mRNA over-expression, not much else is known about CYP6P9 protein expression in *An. funestus*. The role of the CYP6P9 enzyme in the detoxification of varying pyrethroids needs to be investigated to clarify its role in insecticide resistance. With regard to insecticides the rate of insecticide metabolism by P450 enzymes helps understand how fast- or slow-acting these enzymes are, and any metabolites that may be produced supports the role of the enzyme in the detoxification

process. Elucidating the molecular basis of insecticide resistance greatly facilitates resistance management by enabling questions on the origin, spread and impact of resistance to be assessed (Wondji *et al*., 2009). Investigations will aid researchers in the design of better vector control tools and effective insecticides that are also cost effective and suitable for use in malaria stricken areas in southern Africa.

1.7 Hypothesis

Deltamethrin exposure leads to increased transcription of CYP6P9, CYP6P13 and COI in *Anopheles funestus*. Due to its similarity with CYP6P9, CYP6P13 is predicted to have a similar mRNA expression profile to CYP6P9 following pyrethroid exposure. CYP6P9 in resistant strains is an efficient metabolizer of the pyrethroid insecticide permethrin. Increased metabolism and kinetic activity is a resistance response to pyrethroid toxins and the resistant strain (FUMOZ-R) is more efficient at metabolism than the susceptible (FANG) strain.

Protein analysis will help correlate CYP6P9 mRNA expression with protein abundance; however it is not possible to predict overall protein abundance levels in *An. funestus* strains (baseline, susceptible and resistant) based on the P450 activity or mRNA profiling, due to post-translational modifications. It is expected that the resistant strain (FUMOZ-R) will show an increase in protein abundance following exposure to a pyrethroid insecticide. Up-regulation of protein synthesis would suggest CYP6P9 protein is inducible following pyrethroid exposure and overall results would further implicate CYP6P9 as being involved in P450-mediated resistance in this species.

1.8 Objectives

- Determine pyrethroid insecticide susceptibility in four laboratory-reared *An. funestus* strains.
- Investigate oxidative metabolism via the cytochrome P450 assay, using 7-ethoxycoumarin as substrate and compare levels of P450 activity across pyrethroid resistant (FUMOZ-R), susceptible (FANG) and unselected (FUMOZ) *An. funestus* strains.
- Study the effect of deltamethrin exposure on the mRNA expression of CYP6P9, CYP6P13 and COI in the pyrethroid resistant strain (FUMOZ-R).
- Determine if recombinant CYP6P9 enzyme in FUMOZ-R is able to metabolise the pyrethroid permethrin and how efficiently it does so.
- Compare differences in pyrethroid (permethrin) metabolism between resistant and susceptible *An. funestus* strains. This involves cloning and expression of CYP6P9 (FANG) into *Escherichia coli.*
- Design an antibody targeted against CYP6P9 in *An. funestus* and study the abundance of CYP6P9 protein and compare against or relate to mRNA findings.