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# Detoxification enzymes associated with insecticide resistance and exposure to entomopathogenic fungi in Anopheles arabiensis

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# Detoxification enzymes associated with insecticide resistance and exposure to entomopathogenic fungi in *Anopheles arabiensis*

Luisa Nardini

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

Johannesburg, 2012

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

……..

……. day of ……, 2012

"He had a fever…

And when the fit was on him, I did mark

How he did shake"

William Shakespeare

Dedicated to all the African children who have died from, or been affected by malaria.

# Publications and presentations arising from this study

## Publications

**Nardini, L.**, Christian, R.N., Coetzer, N., Ranson, H., Coetzee, M., L.L. Koekemoer, 2012. Detoxification enzymes associated with insecticide resistance in laboratory strains of *Anopheles arabiensis* of different geographic origin. Parasit. Vectors 5: 113. *(see Appendix A)*

## Conference presentations

2012 **L. Nardini**, M. Coetzee, L.L. Koekemoer. An evaluation of genes associated with DDT resistance and exposure to *Beauveria bassiana* in the major African malaria vector, *Anopheles arabiensis*. 23rd Congress of the South African Society of Biochemistry and Molecular Biology/Federation of African Societies of Biochemistry and Molecular Biology

2009 **L. Nardini**, M. Coetzee, L.L. Koekemoer. Detoxification enzymes associated with DDT resistance in populations of *Anopheles arabiensis* of different geographical origin. 38th Annual Conference of the Parasitological Society of Southern Africa.

# ABSTRACT

*Anopheles arabiensis* is one of the major African malaria vectors, and DDT and pyrethroid resistance in this species is widespread. The aim of this study was to investigate, in detail, what detoxification enzymes are associated with insecticide resistance using the *An. gambiae* "detox chip", a small-scale microarray based on genes that are putatively involved in metabolic detoxification of insecticides. The first part of the study focused on two DDT and pyrethroid resistant laboratory strains of *An. arabiensis* – one that originated from Sudan, and a second that originated from South Africa. One P450 was over-transcribed in the Sudanese strain, while 20 genes were over-transcribed in the South African strain. The majority of these were P450s although GSTs and redox genes were also over-transcribed. The use of synergist assays indicated that DDT and permethrin resistance were related to the presence of a *kdr* mutation (determined by PCR), while deltamethrin resistance was based on insecticide metabolism. In order to evaluate the role of enzymatic detoxification in permethrin resistance, a permethrin selected strain was used. No *kdr* mutations were present in this strain. Here, 29 genes were over-transcribed. Most of these were CYP genes (55%), followed by redox genes (21%), and GSTs (14%). A certain degree of overlap in the gene over-transcription was observed between the deltamethrin and permethrin resistant phenotypes. These genes are potentially functional against both pyrethroids, while those that differed were possibly more substrate specific. The final part of the study aimed to assess whether genes that are associated with insecticide resistance are also induced in mosquitoes infected with the entomopathogen, *Beauveria bassiana*. Using microarray data, a subset of important insecticide resistance genes was chosen for analysis following fungal infection. This study was based on the use of qPCR

to detect changes in expression. None of the genes that were investigated were over-transcribed suggesting that virulence factors, such as toxins, produced by *B. bassiana* may not be inhibited by genes that are already over-expressed in insecticide resistant mosquito populations. This is promising for biological control and suggests that the fungi are viable alternatives to insecticides.

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ACh | acetylcholine |
| AChE | acetycholinesterase |
| aRNA | antisense RNA |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pair |
| CCE | carboxyl/cholinesterases |
| cDNA | complementary deoxyribonucleic acid |
| $C_q$ | quantification cycle |
| CYP | cytochrome P450 |
| DDT | dichlorodiphenyltrichloroethane |
| DEM | diethyl maleate |
| DEPC | diethyl pyrocarbonate |
| df | degrees of freedom |
| DNA | deoxyribonucleic acid |
| dpi | days post infection |
| DTT | dithiothreitol |
| dUTP | deoxyuridine triphosphate |
| dNTPs | deoxyribonucleotide triphosphate |
| EDTA | ethylenediaminetetraacetic acid |
| EMBL | European Molecular Biology Laboratory |
| FC | fold change |
| GABA | γ-aminobutyric acid |

| | |
|---|---|
| GPX | glutathione peroxidase |
| GSH | glutathione |
| GST | glutathione S-transferase |
| hpi | hours post infection |
| IGS | intergenic spacer region |
| IPTG | isopropylthio-β-galactosidase |
| IRAC | Insecticide Resistance Action Committee |
| IRS | indoor residual spraying |
| *kdr* | knockdown resistance |
| KWAG | *An. arabiensis* from South Africa |
| KWAG-perm | permethrin-resistant *An. arabiensis* from South Africa |
| KZN | KwaZulu-Natal |
| L | litre |
| LB | Luria Bertani |
| Limma | Linear Models in Microarray Analysis |
| LLIN | long-lasting insecticidal nets |
| M | molar |
| $m^2$ | square metre |
| MBN | *An. arabiensis* from South Africa |
| MBN-DDT | resistant *An. arabiensis* from South Africa |
| mg | milligram |
| ml | millilitre |
| MLT | median lethal time |

| | |
|---|---|
| mM | millimolar |
| mRNA | messenger RNA |
| n | sample size |
| NCBI | National Centre for Biotechnology Information |
| ng | nanogram |
| OP | organophosphates |
| P450 | cytochrome P450/oxidase |
| PBO | piperonyl butoxide |
| PCR | polymerase chain reaction |
| pmol | picomole |
| PMT | photo-multiplier tube |
| Poly (dA) | polydeoxyadenylic acid sodium salt |
| *rdl* | resistance to dieldrin |
| rDNA | ribosomal DNA |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| rpm | revolutions per minute |
| qPCR | quantitative real-time PCR |
| SD | standard deviation |
| SENN | *An. arabiensis* from Sudan |
| SENN-DDT | resistant *An. arabiensis* from Sudan |
| SOD | superoxide dismutase |
| TBE | tris-borate-EDTA |
| TIGR | The Institute for Genomic Research |

| | |
|---|---|
| TPX | thioredoxin peroxidase |
| WHO | World Health Organization |
| X-GAL | 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside |
| µg | microgram |
| µl | microlitre |
| µM | micromolar |

# Chapter 1

# General Introduction

## 1.1 Malaria

About 216 million cases of malaria were reported globally in 2010 (WHO, 2011). Of these, about 655,000 cases resulted in death, and most of these deaths occurred in sub-Saharan Africa where young children and pregnant women are worst affected (WHO, 2011). Infection with *Plasmodium falciparum* during pregnancy is responsible for about 200,000 infant deaths due to stillbirths, premature birth and limited fetal growth (Schwarz et al., 2008). In cases of severe malaria, more than 15% of patients that survive infection remain with serious neurological problems or mild forms of brain damage (Holding and Snow, 2001; Samba, 2001).

The severity of malaria in Africa is related to many factors. These include the presence of well adapted vectors, human activities (for example, deforestation, agriculture and urbanisation), poor healthcare due to socio-economic factors and political instability (Mouchet et al., 1998; Hougard et al., 2002). Malaria transmission is further enhanced through the mass movement of infected people into uninfected areas and "occupational activities" like mining which bring humans and vectors into contact (Mouchet et al., 1998). The situation is exacerbated by drug and insecticide resistance in the *Plasmodium* parasites (Phillips, 2001) and anopheline vectors respectively (Brown, 1986; Coetzee, 2004). Vector control is a major part of malaria control and prevention (WHO, 2011) and as a result, the development of insecticide resistance in vector populations poses significant problems for malaria control (Coetzee, 2004).

Malaria is caused by *Plasmodium* parasites, and is transmitted by certain species of anopheline mosquito. There are five types of human malaria parasites namely *Plasmodium falciparum*,

*P. vivax, P. malariae, P. ovale* and *P. knowlesi* (White, 2008; WHO, 2011). The most common species are *P. falciparum* and *P. vivax*, with *P. falciparum* being the most dangerous due to its clinical manifestation (WHO, 2011). In Africa, *P. falciparum* is the most prevalent and is transmitted mainly by members of the *Anopheles gambiae* complex and the *An. funestus* group. *Anopheles gambiae s.s.* Giles, *An. arabiensis* Patton and *An. funestus* Giles are the most important and widespread vectors (Gillies and Coetzee, 1987).

### 1.2 The *Anopheles gambiae* complex

Members of the *An. gambiae* complex are sibling species (Mattingly, 1977) that cannot be distinguished morphologically but have distinct genetic and behavioural differences which are reflected in their ability to transmit malaria (della Torre et al., 2002). As it stands, the *An. gambiae* complex contains six named species (Gillies and Coetzee, 1987), one unnamed species (Hunt et al., 1998) and a number of West African incipient species within *An. gambiae s.s.* (Fanello et al., 2003). The seven species of the *An. gambiae* complex have been assigned the following names: *An. gambiae s.s.* and *An. arabiensis*, two of the most important vectors in Africa; *An. merus* Dönitz, *An. melas* Theobald, and *An. bwambae* White, minor vectors; and *An. quadriannulatus* Theobald species A and B, which are non-vectors.

*Anopheles gambiae* and *An. arabiensis* are widespread throughout sub-Saharan Africa but *An. gambiae* is more prevalent in the humid areas, while *An. arabiensis* prefers drier environments (Coluzzi et al., 1979; Fontenille and Simard, 2004). Where the species occur in sympatry, predominance of one species over the other is dependent on seasonal changes (Coluzzi et al., 1979), e.g. rainfall will favour *An. gambiae* (Charlwood and Edoh, 1996). *Anopheles melas*

and *An. merus* breed in saltwater. The former is found along the western coast of Africa (and extends as far as Senegal where it is distributed country-wide), while the latter occurs along the eastern side of the continent and on Madagascar, and has been found far inland in Zimbabwe, Mozambique, South Africa and Kenya (Sinka et al., 2012). *Anopheles bwambae* is found only in Uganda in hot springs of the forested areas of Bwamba County (White, 1985). *Anopheles quadriannulatus* A and B are largely cattle feeders and occur in southern Africa and Ethiopia respectively (Hunt et al., 1998; Coetzee et al., 2000).

The different mosquito species can be identified using the polymerase chain reaction (PCR) assay or with the use of chromosomal inversion polymorphisms. PCR for species identification is based on the intergenic spacer (IGS) region of their ribosomal DNA (rDNA) (Scott et al., 1993; Townson and Onapa, 1994; Fettene and Temu, 2003). The technique is particularly useful because it can be carried out with little starting material (i.e. the leg of a single mosquito). Banding patterns of the giant polytene chromosomes, found in larval salivary glands and ovarian nurse cells, have also proven important for identification of members of the *An. gambiae* complex (Coluzzi and Sabatini, 1967, 1968; Hunt, 1973). One can either compare the chromosomes of a single mosquito, band-for-band, with those of a reference map; or one can examine the chromosomes of offspring of an unknown species, crossed with a known species, and observe the presence of asynapsis and heterozygous inversions (Davidson and Hunt, 1973). These techniques require considerable expertise and are time-consuming. Morphological characteristics for species identification have very little use (Coetzee, 1986; Lounibos et al., 1999) and therefore PCR is the preferred method of identification.

### 1.2.1 *An. arabiensis*

*Anopheles arabiensis* is the focus of the present study. This species is unusual in that it can be found resting both indoors and outdoors and is both zoophilic and anthropophilic (Gillies and Coetzee, 1987). Generally, it is absent from the forest regions and more humid areas of West and Central Africa. *Anopheles arabiensis* is the presumed vector of malaria in South Africa in the absence of the other two vector species, *An. gambiae* and *An. funestus* (Gericke et al., 2002). A detailed distribution map of this species is shown in Figure1.1. Larval biology of *An. gambiae* and *An. arabiensis* is similar in terms of their use of transient rain pools, or more fixed locations such as rice fields or swamps, and larvae of both species can be found in the same breeding site (Gillies and Coetzee, 1987).

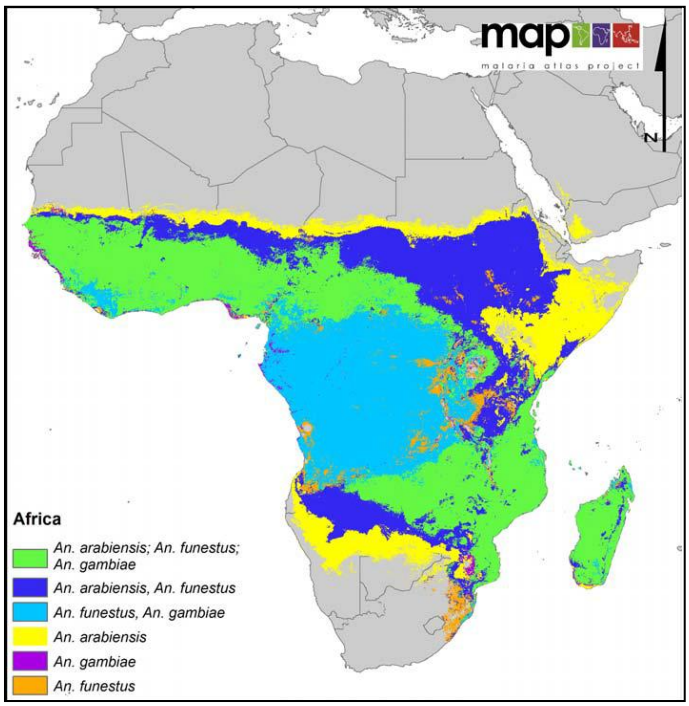



**Figure 1.1** A distribution map of *An. arabiensis* and the other important African malaria vectors, *An. gambiae* and *An. funestus* (from Sinka et al., 2012).

### 1.3 Vector control

The use of long-lasting insecticidal nets (LLIN) and indoor residual spraying (IRS) with WHO approved insecticides are two of the most common ways of preventing transmission of malaria (WHO, 2011). LLINs and IRS work by reducing contact between mosquitoes and humans, and by reducing the life-span of female mosquitoes so that they are unable to transmit *Plasmodium* (WHO, 2011). These approaches are effective only if high coverage is achieved and sustained (WHO, 2011). At present, 12 insecticides in 4 classes are recommended by WHO for IRS (Table 1.1). Pyrethroids are the only class of insecticide approved for LLINs due to the fact that they are fast-acting, long-lasting and demonstrate relatively low toxicity to mammals (WHO, 2011). Alphacypermethrin, bifenthrin, cyfluthrin, deltamethrin, lambdacyhalothrin and etofenprox are all pyrethroids used for LLINs (WHO, 2011).

**Table 1.1** Insecticides recommended by the WHO for IRS against malaria vectors (modified from WHO, 2009)

| Class | Insecticide compound | Mode of exposure |
|---|---|---|
| **Organochlorine** | Dichlorodiphenyltrichloroethane (DDT) | Contact |
| **Organophosphate** | Malathion | Contact |
| | Fenitrothion | Contact and airborne |
| | Pirimiphos-methyl | Contact and airborne |
| **Carbamate** | Bendiocarb | Contact and airborne |
| | Propoxur | Contact and airborne |
| **Pyrethroid** | Alpha-cypermethrin | Contact |
| | Bifenthrin | Contact |
| | Cyfluthrin | Contact |
| | Deltamethrin | Contact |
| | Etofenprox | Contact |
| | Lambda-cyhalothrin | Contact |

### 1.4 Insecticide resistance

Given that insecticide use has been the most successful way of controlling mosquitoes, the development of resistance in target populations has a significant impact on vector control, and ultimately on the prevalence of malaria. The WHO (1957) has defined insecticide resistance as the "development of an ability in a strain of an organism to tolerate doses of a toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of the species". Insecticide resistance in mosquitoes was first observed in *Aedes* species to dichlorodiphenyltrichloroethane (DDT) in 1947 (Brown, 1986). Since then, resistance has developed in a range of species to a variety of insecticides. Long-term exposure to particular insecticides over many generations, along with the large population size and high reproductive rate of mosquitoes (12 generations per year are estimated for *An. gambiae*), increases the likelihood of developing resistance in mosquito populations (Barbosa et al., 2011, and references therein). This is compounded by the fact that a small number of synthetic insecticides are available for use in the field (Barbosa et al., 2011).

Insecticide resistance in *An. arabiensis* has been reported in a number of different countries and to a range of insecticides. Specifically, resistance has been reported in West Africa to DDT and dieldrin (Brown, 1986); in southern Africa to DDT (Hargreaves et al., 2003) and pyrethroids (Mouatcho et al., 2009); in Sudan to malathion, DDT, dieldrin and permethrin (Hemingway, 1983; Himeidan et al., 2007; Abdalla et al., 2008); in Ethiopia to DDT, deltamethrin and permethrin (Balkew et al., 2010); and in Madagascar to dieldrin and Mauritius to DDT (Brown, 1986).

Effective management of insecticide resistance is essential and is dependent on extensive and continuous resistance surveillance. This provides scientists with baseline data for choice of insecticide to be used in control programs, allows for early detection of resistance in vector populations, allows for monitoring of control strategies, and assists with the characterisation of vector populations. Due to the increased reports of insecticide resistance, alternative control interventions such as entomopathogenic fungi (discussed in more detail in section 1.6) are being investigated. However, until additional functional control interventions are available, it is important to understand the mechanisms of chemical insecticide resistance and how resistance develops to aid in resistance management as well as understanding the complex biology of malaria mosquitoes.

**1.5 Modes of insecticide resistance**

According to Hemingway and Ranson (2000), resistance mechanisms are either metabolic (changes in detoxification enzyme activity that lead to detoxification of the insecticide), or target site dependent (i.e. changes in sensitivity of the target site, for example, mutations in the sodium channel genes). Furthermore, these mechanisms can occur together in an organism or population. Increased enzymatic detoxification is considered to be the most common resistance mechanism (Oppenoorth, 1984; Price, 1991; Scott, 1999), although both kinds of resistance have contributed significantly to the rise in insecticide resistance (Hemingway and Ranson, 2000). Another resistance mechanism is based on changes in behaviour by the target insect (behavioural resistance) in the presence of a particular insecticide. For example, DDT causes behavioral changes in mosquitoes by decreasing the rate of mosquito entry into houses, increasing the rate of early exit from houses, and by causing a shift in biting times (Pates and

Curtis, 2005). Reduced penetration of insecticides due to the formation of thicker cuticles is also considered to be an evolved mode of resistance (Karunaratne, 1998). All methods of resistance are under genetic control. The major biochemical mechanisms that confer resistance to the major classes of insecticide are outlined in Figure 1.2.

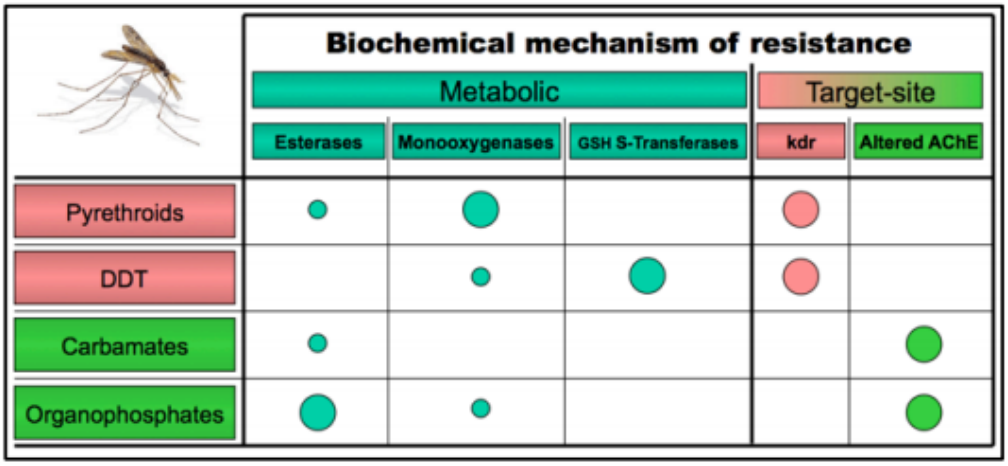



**Figure 1.2** Important biochemical mechanisms that confer resistance to the major classes of insecticides in adult mosquitoes (dot size gives the relative impact of the mechanism on resistance). GSH = glutathione, *kdr* = knockdown resistance, AChE = acetylcholinesterase (IRAC, 2011).

### 1.5.1 Metabolic resistance mechanisms

Metabolic pathways play an important role in insecticide detoxification (Hemingway and Ranson, 2000). There are three enzyme families that are involved in metabolic-based insecticide resistance namely the cytochrome P450s, the glutathione S-transferases (GSTs) and the esterases (Ranson et al., 2002). However, each of these enzyme families is encoded by supergene families and in many cases, the identity of the individual genes involved in

insecticide resistance have not yet been determined (David et al., 2005). These enzyme superfamilies are discussed in greater detail below.

#### 1.5.1.1 Cytochrome P450 monooxygenases

The cytochrome P450s represent one of the largest superfamilies of enzymes and play a number of important roles in living organisms. They are associated with the regulation of hormones, fatty acids and steroids which are central to growth, development and reproduction; and are able to metabolise and detoxify a range of chemicals such as pesticides, plant toxins and drugs (Feyereisen, 1999; Scott, 1999). The cytochrome P450s (named because of the absorption band at 450nm of their carbon-monoxide bound form) are named with the abbreviation 'CYP' (Werck-Reichhart and Feyereisen, 2000). Gene families (more than 40% amino-acid sequence identity) are assigned numbers, subfamilies (more than 55% sequence identity), if present, are indicated by letters, and each gene is assigned a number (Gonzalez and Nebert, 1990; Werck-Reichhart and Feyereisen, 2000). The fact that P450s are present in bacteria, eukaryotes and plants suggests that the P450s are an ancient enzymatic system (Scott and Wen, 2001). According to Feyereisen (1999), about 100 different P450s are encoded for in every insect species (Table 1.2), and all of these have evolved from a common ancestral gene.

**Table 1.2** The number of P450s, excluding pseudogenes, present in the genomes of different insect species (modified from Scott, 2008)

| **Insect** | **Number of P450s** |
|---|---|
| *Anopheles gambiae* | 105 |
| *Aedes aegypti* | 164 |
| *Drosophila melanogaster* | 84 |

The P450s have a broad range of substrate specificity where some P450s can metabolise up to twenty substrates while others can metabolise only one (Scott, 1999). Furthermore, some of the P450s can metabolise the same compounds, and some P450s can function on multiple sites of certain substrates, and produce a range of metabolites (Scott, 1999). Some P450s are produced at all life stages of an insect, while others are stage specific, like *CYP6Z1*, which is produced in adult *An. gambiae* (Scott et al., 1998; Hemingway et al., 2004). Generally, P450s cannot be detected during the egg stage, increase and decrease during the larval stages, are once again undetectable during the pupal stage, and are present at high levels during the adult stage (Scott, 2008). They are also diverse in terms of where they occur in the insect – some P450s occur throughout the organism, while others are restricted to certain organs (Scott et al., 1998). The highest P450 activity is usually associated with the midgut, fat bodies and Malpighian tubules. One P450, *CYP6L1*, is sex-specific and is only expressed in the male *Blattella germanica* (Wen and Scott, 2001).

Cytochrome P450s use molecular oxygen for oxidation. The enzyme inserts one oxygen atom into the substrate (S), and reduces the second oxygen atom to a water molecule using electrons that are provided by NADPH (with the use of reductase). As only one oxygen atom is present

in the oxidised substrate, the enzymes are called monooxygenases (Meunier et al., 2004). This is simplified by the following reaction (from Bergé et al., 1998):

$$S + NADPH + H^{+} + O_2 \Rightarrow SO + NADP^{+} + H_20$$

The SO complex can be unstable and so a number of reactions can occur that stabilise the product. Such reactions include hydroxylation, epoxidation and dealkylation reactions as well as other oxidation reactions (Bergé et al., 1998). The above reaction represents the classical stoichiometry of a P450 reaction but in reality, such a reaction occurs in tightly coupled reactions. Most reactions are uncoupled and superoxide and hydrogen peroxide are produced by the collapse of electron reduced P450-dioxygen complexes (Feyereisen, 1999).

Cytochrome P450-dependent resistance is generally based on increased expression of P450 genes (Hemingway et al., 2004). Enhanced cytochrome P450 monooxygenase transcription is viewed as the most common form of metabolic-based insecticide resistance (Scott, 1999) and their role in detoxification has been reported for almost all classes of insecticides (Ranson et al., 2002). Although the action of P450s typically causes detoxification, they also cause the activation of some substrates (Scott and Wen, 2001). For example, organophosphates (OPs) require activation by P450s and can then be detoxified by GSTs (Hemingway et al., 2004).

#### 1.5.1.2 Glutathione S-transferases (GSTs)

Like the cytochrome P450s and the esterases, the GSTs are a large family of enzymes. They have both enzymatic and non-enzymatic functionality. Their enzymatic capacity is largely responsible for detoxification of endogenous compounds and xenobiotics through a range of mechanisms that include glutathione (GSH) conjugation, dehydrochlorination, GSH peroxidase activity or passive binding (Che-Mendoza et al., 2009). Their non-enzymatic roles are linked to intracellular transport of hydrophobic ligands, signal transduction pathways and protection against oxidative stress (Che-Mendoza et al., 2009).

GST proteins are dimers, and each subunit has an N-terminal and C-terminal joined by a variable linker region. Within these terminals are two binding sites, a G site and an H site (Ding et al., 2003). The G site, present in the N-terminal domain, binds the endogenous tripeptide, GSH, which can be described as an important cellular redox buffer (Schafer and Buettner, 2001; Ding et al., 2003; Che-Mendoza et al., 2009). The H site is the substrate binding site and is consequently more variable. This is present in the larger C-terminal domain which is made up of alpha helices. The H site is hydrophobic and the variability that is observed in this region allows GSTs to have specificity for a range of electrophilic substrates (Che-Mendoza et al., 2009).

The GSTs can be broadly classified as being microsomal (membrane-associated) or cytosolic (occur in the cytoplasm) depending on their location in the cell (Enayati et al., 2005; Che-Mendoza et al., 2009). The microsomal and cytosolic GSTs of insects are very different in

origin, size and structure but catalyse similar reactions (Che-Mendoza et al., 2009; Enayati et al., 2005).

Formal classification of the GSTs was initially based on amino acid sequence data but the criteria for classifying GSTs have expanded to include immunological and structural characteristics, phylogenetic relationships, and gene size and exon/intron structure (Sheehan et al., 2001; Hemingway et al., 2004). Mammalian GSTs have been divided into Alpha, Mu, Pi, Theta, Sigma, Zeta, Kappa and Omega (Sheehan et al., 2001). In insects, the GSTs have been classified as Delta, Epsilon, Omega, Sigma, Theta and Zeta. The largest subfamilies, Delta and Epsilon are specific to insects and are thought to be the main classes associated with detoxification of xenobiotics (Ding et al., 2005). Initially, Delta, Omega and Epsilon were referred to as Class I, II and III, but these were changed to the Greek nomenclature used for the mammalian system. GSTs are named according to the host from which they are isolated, followed by the GST class and then by a number that indicates their order of discovery or genome organisation (Che-Mendoza et al., 2009). For example, the 12th member of the *An. gambiae* Delta class of GSTs to be identified is known as *AgGSTd12* (Hemingway et al., 2004).

GSTs play an important role in insecticide resistance. This is based on increased rates of detoxification or by preventing damage from oxidative stress (Ding et al., 2005). GSTs are responsible for DDT resistance by the dehydrochlorination of DDT (*GSTe2* in *An. gambiae*), and OP resistance is dependent on O-dealkylation or O-dearylation of the insecticide (Ding et al., 2005). GSTs also function by catalysing the secondary metabolism of OPs following P450 activation (Hemingway et al., 2004). Increases in enzyme activity are either a result of an

increase in the amount of GST enzymes due to gene amplification, or as a result of increased rates of transcription (Hemingway et al., 2004).

The molecules, $O_2^{\cdot -}$, –OH and $H_2O_2$, are derived from both endogenous and exogenous sources, are highly reactive and can damage DNA, RNA and proteins (Ahmad and Pardini, 1990). They can also cause lipid peroxidation which is serious in insects as lipids are an integral part of the cell membrane, and have specialised physiological activity (Ahmad and Pardini, 1990; Gaikwad et al., 2010). Peroxides can damage cells directly, or their breakdown products can be harmful. GSTs and GSH have been shown to be very important in prevention and repair of oxidative damage which is caused, amongst other things, by exposure to pyrethroids (Parkes et al., 1993; Kostaropoulos et al., 2001; Vontas et al., 2001). This can occur passively by sequestration (Kostaropoulos et al., 2001), or actively by peroxidase activity (Vontas et al., 2001).

#### 1.5.1.3 Carboxyl/cholinesterases (CCEs)

The carboxyl/cholinesterases (CCEs) perform a range of physiological functions. They are involved in the breakdown of neurotransmitters, metabolism of certain hormones and pheromones, detoxification, defence and behaviour (Taylor and Radić, 1994). In insects, they play an important role in protection against insecticides, in particular resistance to OPs and to a lesser degree, in carbamate and pyrethroid resistance (Hemingway and Karunaratne, 1998, and references therein).

CCEs, along with acetylcholinesterases (AChEs), belong to the carboxyl/cholinesterase superfamily. The function of these enzymes is based on the activity of a highly conserved catalytic triad that generally includes Ser-His-Glu and less often, Ser-His-Asp (Oakeshott et al., 1999). In addition, there are three specific locations on the enzyme that accommodate the alcohol, acid and oxyanion moieties of their substrates (Oakeshott et al., 1999). Catalysis of the substrate is a two-step process. The first step releases the alcohol moiety from the substrate and a covalent bond forms between the Ser and the remaining acid moiety (Oakeshott et al., 1999). The second step results in the cleavage of the bond and releases the acid moiety of` the substrate with the Ser (Oakeshott et al., 1999).

The CCEs confer resistance in two main ways: by an increase in production of the enzymes, or by mutations in the genes that encode the enzymes (Cui et al., 2011). In the former instance, high levels of esterases are produced, either by transcriptional over-expression, or by gene amplification (Cui et al., 2011). This allows for sequestration of insecticides i.e. rapid binding and slow turnover of insecticide by the enzyme, despite the fact that the carboxylesterases have extremely limited OP hydrolytic activity (Oakeshott et al., 1999). Sequestration confers resistance to OPs and carbamates (Hemingway et al., 2004). In addition to sequestration, mutational changes in the enzymatic properties of non-specific esterases occur so that they exhibit increased activity toward OPs (OP hydrolase activity) (Oakeshott et al., 1999). This occurs through point mutations in the structural genes (Hemingway et al., 2004). CCE-based resistance also involves a mutation in the main target of OPs and carbamates, namely AChE, a highly specific esterase (Cui et al., 2006). These are structural changes in the enzymes and are discussed in greater detail in the relevant sections.

The CCEs have been divided into eight subfamilies. These are α-esterases, juvenile hormone esterases, β-esterases, gliotactins, AChEs, neurotactins, neuroligins and glutactin type. The juvenile hormone esterases and α- and β-esterases are the largest subfamilies and form one large ancestral clade (Ranson et al., 2002). The remainder of the subfamilies are cell surface proteins and are non-catalytic. In terms of sequence homology, they resemble AChE most closely (Ranson et al., 2002).

### 1.5.2 Target site resistance

A number of target-site based resistance mechanisms have been investigated. These include '*kdr*' or knockdown resistance – mutations in the voltage gated sodium channel that confer resistance to pyrethroids and DDT; modified AChE, leading to OP and carbamate resistance; and a mutation in the γ-aminobutyric acid (GABA)-gated chlorine channel leading to resistance to chlorinated hydrocarbons (Hemingway et al., 2004). Target site resistance has been reviewed in detail by a number of authors including Price (1991), ffrench-Constant (1999), Zlotkin (1999) and Hemingway et al. (2004).

#### 1.5.2.1 Knockdown resistance (*kdr*)

Pyrethroids and DDT target the voltage gated sodium channel (Soderlund and Bloomquist, 1989; Zlotkin, 1999). The sodium channel is a transmembrane structure of four homologous domains (I-IV) and each domain is made up of six helical segments (S1-S6) (Figure 1.3) (ffrench-Constant, 1999; Zlotkin, 1999). Nerve impulses depend on a wave of transient

membrane depolarisation, known as an action potential, that passes along a nerve cell (Voet and Voet, 1995). Action potentials result from the presence of voltage gated channels which allow for ion-specific permeability changes across a membrane (Voet and Voet, 1995). During resting potential of the membrane, the channel is in an inactive state and is therefore closed but upon channel activation the membrane becomes depolarised, causing the channel to open by generating a sodium current. The channel stays open for a few milliseconds after which inactivation occurs and the movement of ions across the membranes is blocked (i.e. the channel is still open, but due to a conformational change in the channel, ions cannot traverse). When the membrane potential returns to the resting level, the channel closes. The sodium channels function in concert with other pumps e.g. potassium, sodium-potassium, that help to restore the original electric potential of the cell (Martins and Valle, 2012).

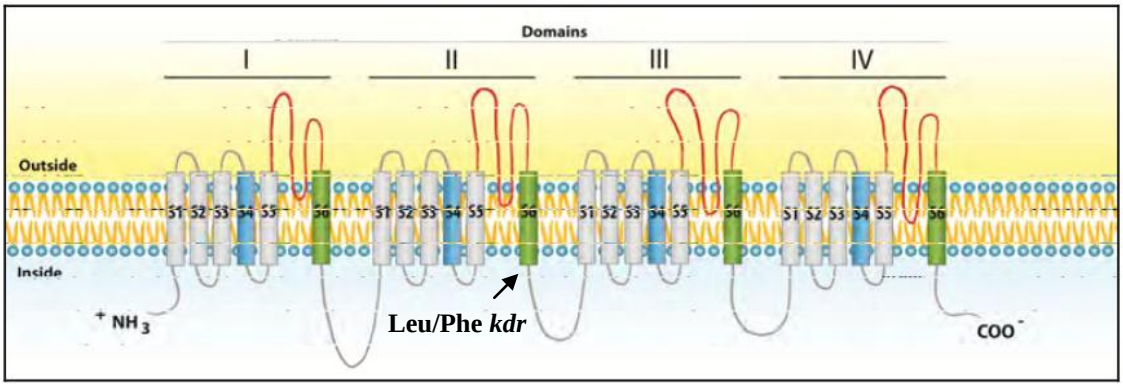



**Figure 1.3** The voltage gated sodium channel comprising four homologous domains (I-IV) and the six helical segments of each domain (S1-S6) (Martins and Valle, 2012). The East and West African mutations occur in Domain II, segment 6 (indicated on figure).

Depending on their chemical structure, pyrethroids are classified as type I or type II (Figure 1.4) and the former are the more toxic of the two (Narahashi, 1992). Pyrethroids slow both channel activation and inactivation. In addition, they extend the time of channel opening (channels can remain open for up to a few seconds) (Narahashi, 1992). This means that sodium ions flow through the channel continuously causing membrane depolarisation and consequently hyperactivity of the nervous system, paralysis and death. Insecticides cannot bind to their target site in the channel when it is closed, but when the channel is open, DDT and pyrethroids bind and alter the gating kinetics of the system (Martins and Valle, 2012).

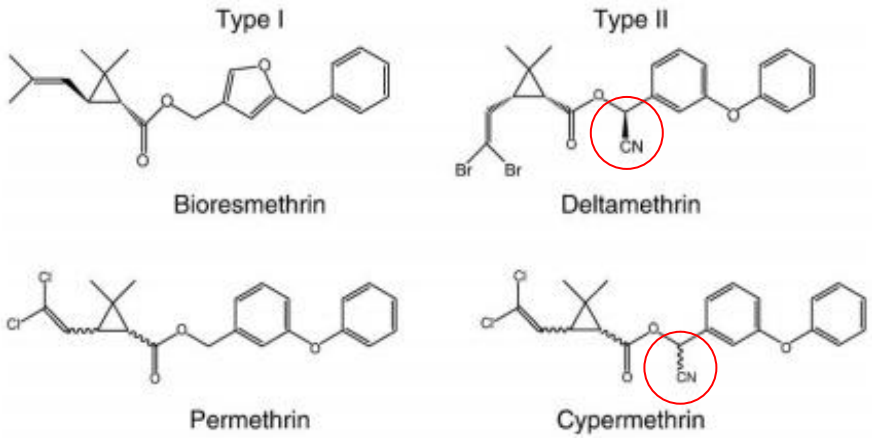



**Figure 1.4** The chemical structure of type I (left) and type II (right) pyrethroids. They differ in terms of the presence of an α-cyano (CN) group at the phenylbenzyl alcohol position. In type I, this is absent (modified from Du et al., 2009).

Knockdown resistance was first observed in DDT-resistant houseflies (Milani, 1954; cited in Bass et al., 2007) and much later, it was reported that this was due to a single amino acid mutation in the S6 segment of domain II (Williamson et al., 1996). Since then, such mutations

have been identified in a number of different insects and result in diminished binding ability between toxin and sodium channel (Scott and Dong, 1994; Park and Taylor, 1997; Liu et al., 2000; Franck et al., 2012). In *Anopheles*, two mutations exist. One is known as the West African mutation and results in a change from leucine to phenylalanine (L1014F) of the S6 segment (Martinez-Torres et al., 1998). The East African version occurs at the same codon but is a leucine to serine mutation (L1014S) (Ranson et al., 2000). Although the naming of the mutations (East and West) was based initially on where they were found, studies have since shown that the mutations are not restricted to these areas. In addition, both mutations have been found in one population (Verhaeghen et al., 2006). A number of studies have shown that *kdr* is responsible for resistance in many populations of the major African malaria vectors, *An. gambiae* and *An. arabiensis* (Diabaté et al., 2004; Santolamazza et al., 2008; Yewhalaw et al., 2011) and in other mosquitoes of medical importance, such as *Ae. aegypti* and *An. stephensi* (Brengues et al., 2003; Enayati et al., 2003; Chang et al., 2009).

#### 1.5.2.2. Other target site mutations

##### 1.5.2.2.1 Acetylcholinesterase (AChE)

Acetylcholine (ACh) is a neurotransmitter, relaying nerve impulses across synapses. AChE is a fast-acting enzyme that hydrolyses ACh so that nerve impulses are terminated (Hemingway et al., 2004). AChE is the target of OP and carbamate insecticides which covalently bind to the active site and prevent normal function of the enzyme (Fournier et al., 1992). In OP- and carbamate-resistant insects, AChE is modified by point mutations in the *ace-1* gene (called *ace-1*$^{R}$) that result in amino acid residue changes which effectively alters the orientation of

active site residues (Vontas et al., 2002; Hemingway et al., 2004). In mosquitoes, insensitive AChE is generally characterised by high levels of carbamate resistance (but carbamate resistance is not always due to insensitive AChE, e.g. Oduola et al., 2012), and low levels of OP resistance (Hemingway et al., 2004; Russell et al., 2004).

#### 1.5.2.2.2 GABA receptors

γ-Aminobutyric acid (GABA) is also a neurotransmitter, but functions in an inhibitory capacity i.e. to inhibit signals (Holum, 1998), where binding of GABA to its receptor results in gating of chloride ion channels (Hosie et al., 1997). GABA receptors comprise five different subunits that surround a "transmitter-gated ion channel" (Hosie et al., 1997). The subunits are encoded for by the *rdl* (resistance to dieldrin) gene and mutations in this gene confer resistance to cyclodienes (ffrench-Constant, 1999). Interestingly, the same amino acid substitution in the *rdl* gene in numerous insects of different orders confers resistance. The unique alanine 302 substitution with serine confers resistance by blocking the insecticide binding site, and also by destabilising the site (ffrench-Constant, 1999).

### 1.5.3 Behavioural resistance

According to Pates and Curtis (2005), behavioural resistance may occur as a physiological response in the presence of an insecticide, or it may be under genetic control. Some insecticides (like DDT and pyrethroids) are irritating to mosquitoes – the term "excito-repellency" describes a disturbance of resting mosquitoes and an increase in flight activity of

mosquitoes, or avoidance of a surface covered with insecticide (Chareonviriyaphap et al., 1997). This means that mosquitoes may not acquire a lethal dose of insecticide, despite the use of IRS or LLINs. This effect is related to both the insecticide and the formulation of the insecticide (Takken, 2002). Behavioural resistance is particularly interesting in the context of LLINs. It has been shown that the presence of insecticide treated bednets in houses does not affect the number of mosquitoes that enter the house, but their presence does increase the number of mosquitoes that are unfed and the number of mosquitoes that exit the house (Takken, 2002 and references therein). It is thought that in such instances, mosquitoes may change their biting behaviour where they bite at different times or will bite people outside; and they may also change host preference (Takken, 2002).

### 1.5.4 Cuticular resistance

Cuticular thickening has been reported in a number of insecticide-resistant insects (Gunning et al., 1995; Pan et al., 2009; Wood et al., 2010; Lin et al., 2012) and is thought to aid resistance by slowing the penetration of insecticides (Pan et al., 2009). Cuticle thickening is associated with higher protein and lipid content or greater sclerotisation is observed (Pan et al., 2009 and references therein).

## 1.6 Biological control of mosquito vectors using entomopathogenic fungi

Biological control of insects has gained importance in recent years due to increased pressure to reduce the use of chemical insecticides and their residues in the environment and food. The development of resistance to chemical insecticides, and subsequent reduction in insecticide

efficacy, has also encouraged scientists to search for additional control strategies that might be used as part of integrative vector control programs (i.e. to supplement current control strategies). Interest in entomopathogenic fungi as potential mosquito control agents has gained momentum in recent years and fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* provide a natural and environmentally friendly means of vector control (Scholte et al., 2004). Most fungal entomopathogens belong to the Ascomycota and Zygomycota (Hegedus and Khachatourians, 1995; Roy et al., 2006). *Beauveria bassiana* is a soil-borne Ascomycete and belongs to the asexual Hyphomycetes. These are filamentous fungi that reproduce by aerial conidia, attached to conidiophores that grow off the substrate (Scholte et al., 2004). The full classification of some important entomopathogenic Ascomycete fungi can be seen in Table 1.3.

Fungi have been studied extensively as agents for control of agricultural pests. For example, Wraight et al. (2010) have recently assessed the virulence of 43 isolates of *B. bassiana* against eight different lepidopteran pests. *Beauveria* and *Metarhizium* represent the most common commercial mycoinsecticides (Faria and Wraight, 2007). In South Africa, some *Beauveria* based products are available for crop control (e.g. Broadband™, Becker Underwood) and small-scale use (e.g. Bb Plus®, Becker Underwood). South and North America are the largest developers of fungal biological control products (Faria and Wraight, 2007).

**Table 1.3** Classification of the genera of entomopathogenic Ascomycota (abbreviated from Roy et al., 2006)

| **Division** | **Class** | **Order** | **Family** | **Genus** |
|---|---|---|---|---|
| Ascomycota | Sordariomycetes | Hypocreales | Clavicipitacea | *Beauveria*<br>*Cordyceps*<br>*Cordycepioideus*<br>*Lecanicillium*<br>*Metarhizium*<br>*Nomuraea* |

The first documented study of infection of mosquitoes with *Beauveria* was conducted in the 1960s (Clark et al., 1968). Since then, a number of studies have been published showing that *B. bassiana* is able to cause infection and death in a number of mosquito species (Farenhorst et al., 2009; Farenhorst et al., 2010; Kikankie et al., 2010; Blanford et al., 2011; Darbro et al., 2012). Fungal infection usually occurs via the insect cuticle and for this reason they are useful as the insect need not "ingest" the spores in order to become infected (Gupta et al., 1992; Pedrini et al., 2007). Infection is initiated by attachment of a spore to the insect cuticle, the structure and composition of which determine whether the spore will germinate and infect a particular host (Pedrini et al., 2007). In the case of *B. bassiana*, an exogenous carbon source (e.g. chitin and fatty acids) is required (Pedrini et al., 2007, and references therein). Both *B. bassiana* and *M. anisopliae* can germinate in the presence of non-specific carbon sources – a fact that supports their broad host range (Charnley, 1989, and references therein). Following adhesion of conidia to the insect integument, the conidia germinate on the cuticle and produce an infection peg which penetrates the cuticle directly (Gillespie, 1988). The initial location of penetration is often at the joints between segments, and both physical force and enzymatic

degradation are involved in penetration of the cuticle (Charnley, 1989). Chitinases, proteases and lipases are produced, typically in response to the composition of the cuticle as the hyphae progress through the cuticle (Hegedus and Khatchatourians, 1995). It has been shown that a degree of quantitative and qualitative variability is associated with enzyme production by different strains of *B. bassiana* (Gupta et al., 1992). When the hyphae reach the haemocoel, blastospores, generated by the separation of the hyphae at the septa, are produced (Feng et al., 1994). The blastospores are thin-walled infectious cells that multiply by budding and are responsible for transfer of the infection throughout the insect host. Insect death occurs at this phase of the infection and is caused by a combination of toxin production (by the blastospores), physical obstruction of haemolymph circulation, nutrient depletion and the invasion of host tissues (Scholte et al., 2004). *Beauveria* species produce a range of toxins that are mostly low molecular weight, cyclic peptides. Some of these include beauvericin, bassianolide, beauveriolides, bassianin, tenellin and oosporein (Mazet et al., 1994; Strasser et al., 2000). Infection is completed when the blastospores change back to the mycelial form and grow out of the insect producing external conidiophores (Mazet et al., 1994).

It has been shown that fungal infection can increase the activity of detoxification enzymes in *Galleria mellonella* and that fungal infection reduces susceptibility to insecticides (Serebrov et al., 2006). This is significant because, if an insecticide resistant population is to be treated with fungi, the population might be less susceptible to the fungal agents if the same detoxification enzymes are up-regulated following insecticide or fungal exposure. The role of detoxification enzymes in the formation of insect resistance to infectious agents has not been explored in great detail and little published data are available on the effect of insecticides on insect

sensitivity to fungal infections. This is important to consider when testing and evaluating a fungal control agent.

## 1.7 Microarrays

### 1.7.1 An introduction to microarrays

Microarray technology is a valuable tool that has enabled scientists to rapidly assess the expression of thousands of genes (Naidoo et al., 2005). The basic concepts and experimental design considerations have been outlined by Murphy (2002) and Naidoo et al. (2005). Microarray technology is based on the fact that an mRNA molecule is able to bind specifically to the DNA template of its origin (NCBI, 2007). Microarrays are typically prepared on glass slides where cDNA fragments of different genes, amplified by PCR or artificially synthesised oligomers, are spotted in a specific pattern (Karakach et al., 2010). The RNA extracted from two independent samples is transcribed into cDNA and the control and test samples are labeled with the fluorescent dyes, Cy5 (red) and Cy3 (green), respectively. The labeled cDNAs are co-hybridised to the slide which is then washed, and scanned by lasers that activate the dyes. The fluorescent signal is measured for each feature and the relative fluorescent signal of each dye is recorded. The ratio of Cy3:Cy5 corresponds to the relative amount of transcript in the samples. Figure 1.5 illustrates a basic outline of a microarray experiment.





**Control** (e.g. susceptible mosquito)

**Test** (e.g. DDT-resistant mosquito)

RNA extraction

Control and test RNA are reverse transcribed into cDNA and labeled with Cy5 (red) and Cy3 (green) fluorescent dyes respectively

Labeled samples are pooled and hybridised to the array

- Slides are washed and scanned by lasers that excite the fluorescent dyes.
- Each spot produces a fluorescent signal: the ratio of fluorescence produced by the dyes is computed.
- The ratio represents the relative amount of transcript from each sample.

**Red spots (Cy5)** = sample obtained from the control

**Green spots (Cy3)** = sample obtained from the test sample

**Yellow spots** = transcripts present in both samples

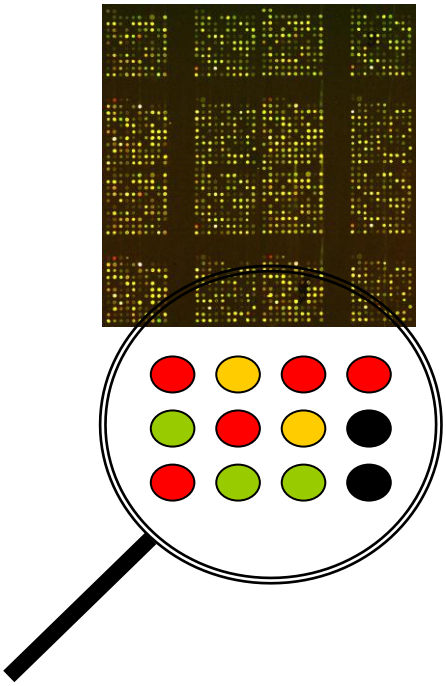


**Figure 1.5** Summary of the general procedure of a microarray experiment.

When comparing gene expression between two samples, two possible approaches are typically used in microarray experiments: these are direct or indirect comparisons (Dombkowski et al., 2004 and references therein). In the former, the test sample (e.g. DDT-resistant mosquito material) is co-hybridised with cDNA derived from the control (DDT-susceptible mosquito). This provides a direct comparison between the two samples (and is called "direct design") (Figure 1.6 A). In the latter, material from different biological sample (e.g. cDNA from 3 biological repeats) is compared with cDNA from a common reference sample, which is used in each microarray that is prepared in the study. This is referred to as a reference design experiment (Figure 1.6 B). Reference designs are useful but can be criticised for introducing variability into microarray data when compared with a direct design set-up (Dombkowski et al., 2004).

As in most biological experiments, replication is required in microarrays. Generally, two types of replicates are used: biological and technical (Naidoo et al., 2005). A biological repeat is where samples from different specimens is obtained. A technical repeat can include multiple spots on one microarray, or preparing two microarrays from a single cDNA sample. A form of technical replication is dye-swaps, although this is usually considered an additional form or replication. This is where in one microarray the control is labeled with Cy3 and the test cDNA with Cy5; and in the next microarray, the control is labeled with Cy5 and the test cDNA is labeled with Cy3 dye. Dye swaps are useful as Cy3 and Cy5 bind to cDNA with different affinities and so bias is avoided by swapping dyes and using data from both experiments.

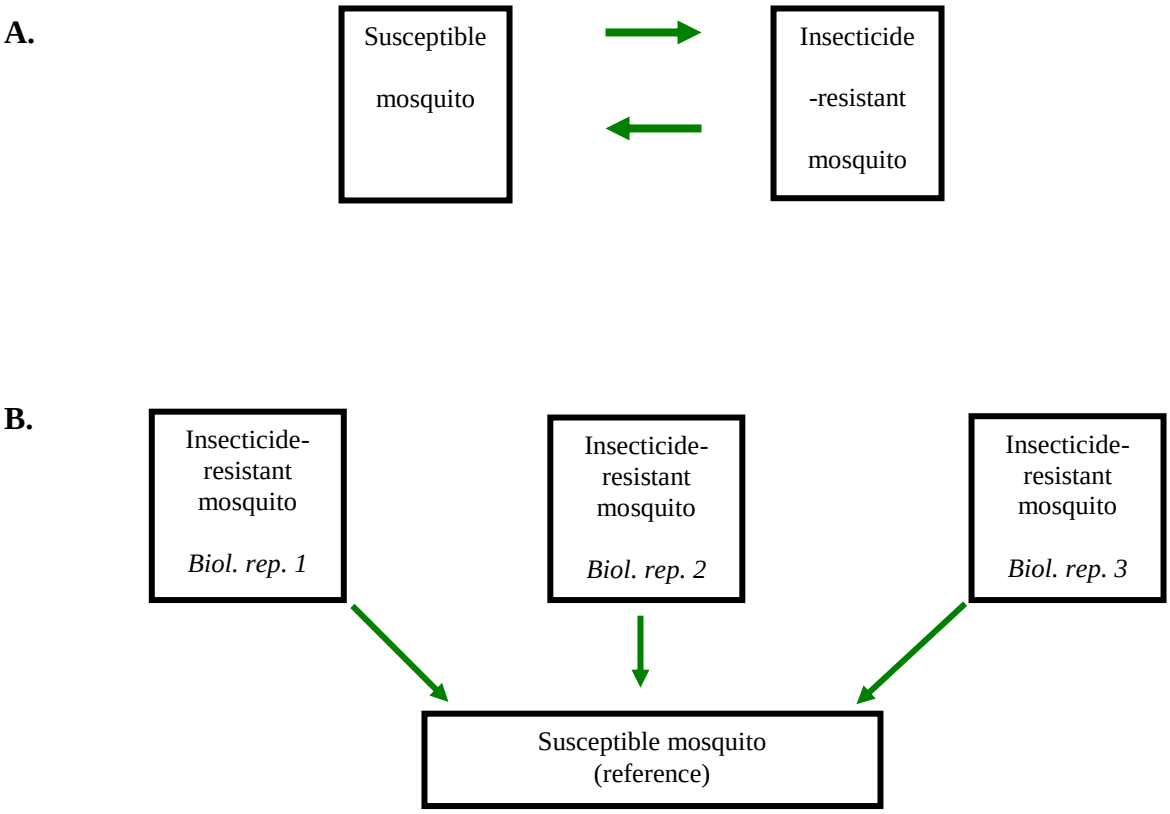



**Figure 1.6** Basic set-up of (A) a direct design and (B) a reference design. Biol. rep. = biological repeat.

### 1.7.2 The *Anopheles gambiae* detoxification chip

An *An. gambiae* "detoxification chip" was developed by the Liverpool School of Tropical Medicine in the United Kingdom. It is a boutique array made up of genes that are thought to be involved in metabolic based insecticide resistance (David et al., 2005). This includes 103 P450s, 31 carboxylesterases, 35 GSTs, 41 Red/Ox genes, 5 ATP–binding cassette transporters, tissue specific genes and housekeeping genes (David et al., 2005). The probes were prepared using PCR or were artificially synthesised as 70-mer antisense oligos. Each probe has been spotted twice on the array and along with the artificial spike-in control genes (Universal

Lucidea Scorecard), a total of 230 spots are on the microarray. The arrays were prepared on gamma-amino-propyl-silane-coated glass slides from Corning. Coating of glass slides alters the surface chemistry to allow for binding of DNA during the spotting process (improves strength of binding as well as uniformity of spots in terms of size, shape and probe concentration) (Corning Incorporated, Life Sciences, 2005).

The *An. gambiae* detoxification chip has been used with success in elucidating genes not previously implicated in insecticide resistant *An. gambiae* (David et al., 2005). Since the original study, the detox chip has also been used for cross-species hybridisations. Müller et al. (2007b) investigated expression profiles of *An. arabiensis* following exposure to pyrethroids using the *An. gambiae* microarray. According to these authors, the performance of the detoxification microarray was similar between *An. gambiae* and *An. arabiensis*. Their conclusions were based on the percentage of probes that were detected after hybridisation. An important benefit of using a boutique array like the detoxification chip is that its use allows researchers to search for differential expression in a defined set of genes, as opposed to searching for differentially expressed genes in an entire genome where the assortment of gene expression changes is likely to be massive. Cross-species hybridisations using the "detox" chip have also been done with *An. stephensi* (Vontas et al., 2007) and *An. funestus* (Christian et al., 2011).

The use of *An. gambiae* detoxification microarray has generated much important and useful information about which enzymes might play a role in insecticide resistance (David et al., 2005; Strode et al., 2006; Müller et al., 2007a, 2007b, 2008; Vontas et al., 2007; Christian et al., 2011). Furthermore, microarray technology has simplified the task of assessing which

genes are involved in a particular phenotype, especially in instances where large enzyme families, such as the P450s, GSTs or CCEs are involved.

### 1.8 Real-time quantitative PCR (qPCR)

As the name suggests, real-time quantitative PCR (qPCR) allows one to monitor the amplification of DNA in real-time and is used for quantifying a specific gene in a sample (Qiagen, 2009). Relative quantification is one type of real-time PCR analysis and is used, amongst other things, for microarray data validation. In relative quantification, the ratio between a target gene and reference gene are determined and the ratio is compared between two experimental conditions/samples/tissues (Qiagen, 2009). A range of potential reference genes are usually tested and the gene with the most stable expression is used for relative quantification. Stable reference genes are those that do not show changes in expression under the conditions one is studying (Bio-Rad, 2006). Generally, a limited number of genes shown to be over-transcribed in a microarray experiment are selected for validation by qPCR (Rockett and Hellmann, 2004).

A basic amplification plot is shown in Figure 1.7. As with conventional PCR, the amount of PCR product approximately doubles with each reaction cycle, and this phase of PCR is known as the exponential phase. The reaction plateaus when reaction components (e.g. dNTPs) become limiting. During the initial stages of PCR, the fluorescence of the product is still very low and cannot be detected. In other words, it is below threshold. However, eventually sufficient amplification occurs so that the product fluorescence increases to the point of

detection. This is known as the quantification cycle or $C_q$ and refers to the cycle at which point this occurs. In large part, the $C_q$ is determined by the amount of starting material.

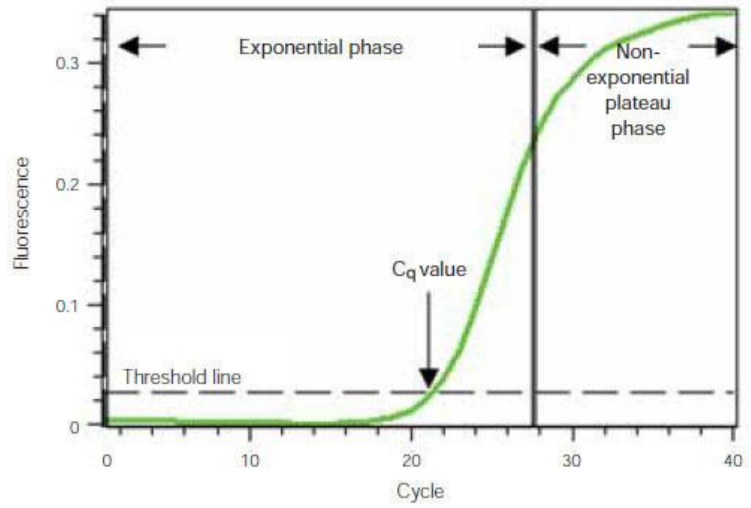



**Figure 1.7** Theoretical amplification plot of a single PCR product (from Bio-Rad, 2006)

Different approaches are available for data analyses. These include the Livak method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001), and the Pfaffl method (Pfaffl, 2001). The Livak method can be used when the amplification efficiency of the reference and target genes are almost the same (and as close to 100% as possible). However, when this is not the case, the Pfaffl method is used to calculate relative expression. The expression ratio between the reference and target genes is calculated by the following formula:

$$\text{Ratio} = (E_{target})^{\Delta Cq,\ target\ (calibrator\ -\ test)} / (E_{ref})^{\Delta Cq,\ ref\ (calibrator\ -\ test)}$$

*Where:*

**Efficiency (E)** is calculated by $E = 10^{-1/slope}$ using the slope of the standard curve.

$\mathbf{E_{target}}$ and $\mathbf{E_{ref}}$ are the amplification efficiencies of the target and reference genes respectively.

$\mathbf{\Delta C_q}$**, target (calibrator-test)** is the $C_q$ of the target gene in the control sample minus $C_q$ of the target gene in the test sample.

$\mathbf{\Delta C_q}$**, ref (calibrator-test)** is the $C_q$ of the reference gene in the control sample minus the $C_q$ of the reference gene in the test sample.

### 1.9 Study Objectives

The overall aim of this study is to investigate the differences in metabolic enzyme transcripts between insecticide resistant and susceptible *An. arabiensis*, and their changes, if any, when mosquitoes are infected with entomopathogenic fungi. The specific objectives of this study are summarised in Figure 1.8. Briefly, they are as follows:

1. To determine which detoxification enzymes are associated with DDT resistance in laboratory strains of *An. arabiensis* (Chapter 2).
2. To compare the detoxification enzyme profiles of DDT- and pyrethroid-resistant *An. arabiensis* from different geographic origins (Chapter 2).
3. To investigate the enzymes associated with permethrin resistance in *An. arabiensis* (Chapter 3).
4. To investigate the impact of exposure to *B. bassiana* on the *An. arabiensis* detoxification profile, and compare this with the detoxification profile associated with insecticide resistance (Chapter 4).

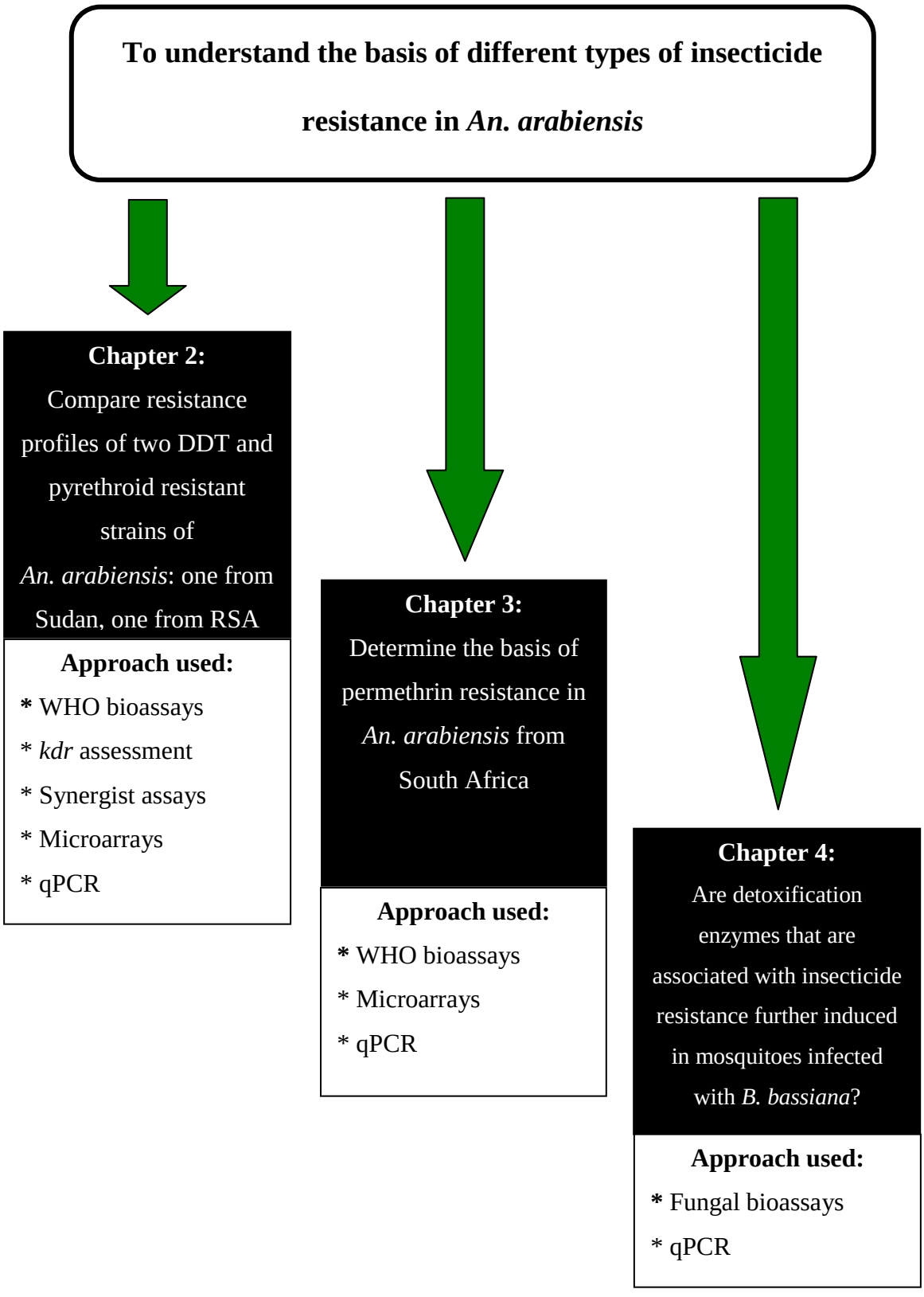



**Figure 1.8** Basic outline of the study and brief description of approach used for each section.

# Chapter 2

## Detoxification enzymes associated with insecticide resistance in laboratory strains of *Anopheles arabiensis* of different geographic origin

## 2.1 Introduction

*Anopheles arabiensis* is one of the major African malaria vectors and belongs to the *An. gambiae* complex. Insecticide resistance in this species is widespread. Examples include dichlorodiphenyltrichloroethane (DDT), deltamethrin and permethrin resistance in Ethiopia (Balkew et al., 2010; Yewhalaw et al., 2011); partial resistance to permethrin in Tanzania (Matowo et al., 2010); DDT, permethrin, malathion and bendiocarb resistance in Sudan (Hemingway, 1983; Abdalla et al., 2008); DDT and permethrin resistance in South Africa (Hargreaves et al., 2003; Mouatcho et al., 2009); and resistance to propoxur in Mozambique (Casimiro et al., 2006).

As explained in Chapter 1, insecticide resistance is commonly based on an increase in levels of detoxification enzymes belonging to large enzyme families, known as super-families. In *An. gambiae* there are multiple cytochrome P450 (n=105), esterase (n=40) and GST genes (n=28) (Strode et al., 2008). Because so many genes are included in these families it is difficult to determine which specific gene(s) are associated with resistance to a particular insecticide, or class of insecticides. The development of high throughput technology such as microarrays provides a solution to this problem (Naidoo et al., 2005). The *An. gambiae* detoxifiction microarray is a custom–made boutique array that includes GSTs, esterases and P450s as well as a number of redox genes that are associated with P450 metabolism and which protect against free radical damage (David et al., 2005). Although the detox chip was constructed using *An. gambiae* sequence information, it has been used with success in a number of cross-species hybridisations with *An. arabiensis* (Müller et al., 2007b), *An. funestus* (Christian et al., 2011) and *An. stephensi* (Vontas et al., 2007).

The aim of this study was to compare the transcription of detoxification enzyme genes of two DDT-selected resistant *An. arabiensis* strains from different geographic locations, one from Sudan and the other from South Africa. In Sudan, vector control includes the use of long-lasting insecticidal nets (LLINs), temephos for larviciding, and bendiocarb is used for IRS (Ranson et al., 2009). South African vector control approaches include the use of IRS with DDT in traditional unplastered mud, reed or wood houses and pyrethroids on walls with enamel painted surfaces (Maharaj et al., 2005; Coleman et al., 2008). LLINs are also recommended for use as a personal protection method, but are not part of the national control strategy (WHO, 2011).

## 2.2 Materials and methods

### 2.2.1 Mosquito strains

Mosquitoes were maintained under standard insectary conditions of 26 ± 2°C, and at a relative humidity of 70-80%, with a 12:12 light:dark cycle and 45 minute dusk/dawn period. Eggs were formalin sterilised (1%) and larvae that hatched were maintained in distilled water and fed on a powdered yeast (Vital Brewer's Yeast) and dog biscuit (West's Beeno; unflavoured, low fat content) mixture (1:3). A 10% sugar (Huletts® White Sugar) solution was provided for adults, and females were provided with three blood meals a week. The strains used for this study were as follows: *An. arabiensis*, colonised in the 1980's from the Sennar region of Sudan (SENN) and *An. arabiensis*, colonised in 2002 from Mamfene in KwaZulu-Natal Province, South Africa (MBN). SENN-base and MBN-base were the susceptible or

"unselected" strains while SENN-DDT and MBN-DDT were the resistant (selected) strains. The susceptible strains were colonised when resistance was originally detected in the field population, but have not been under any laboratory selection on DDT. The resistant strains have been under continuous DDT selection from the time of colonisation. To maintain resistance in the selected strains, three day old adults were exposed to 4% DDT in every generation (see section 2.2.3). Both DDT selected strains from Sudan and South Africa showed very low or no mortality (after 24 hour recovery period) following exposure to DDT for 1 hour and both were homozygous for the L1014F *kdr* mutation (see section 2.2.2). All strains were maintained in separate insectary rooms to minimise the chance of contamination between strains.

### 2.2.2 Detection of *kdr*

#### 2.2.2.1 DNA extraction

DNA was extracted from 25 MBN-DDT and 25 SENN-DDT mosquitoes (males and females). Two legs were removed from each mosquito using sterile forceps, and placed in a sterile 0.2ml reaction tube. Components of the *prep*GEM™ Insect kit (ZyGEM) were added to each tube (17µl nuclease-free water, 4µl 10x Buffer, 1µl *prep*GEM™) and the legs crushed using a sterile pipette tip. Each sample was incubated at 75°C for 15 minutes, and then at 95°C for 5 minutes.

### 2.2.2.2. PCR and sequence analysis

PCR reactions were prepared for each sample (Table 2.1). Cycling conditions were as follows: 94°C/2 minutes, (94°C/30 seconds, 50°C/30 seconds, 72°C/30 seconds) x 40 cycles, and a final extension step at 72°C for 5 minutes. The primers, AGD1 (5' ATA GAT TCC CCG ACC ATG 3') and AGD2 (5' AGA CAA GGA TGA TGA ACC 3'), span the region containing the mutations (Martinez-Torres et al., 1998). Primers were synthesised by Macrogen Korea. Amplified samples were viewed (based on size) by electrophoresis on a 2.5% agarose gel and were sequenced in both directions by Macrogen Europe. Sequences were aligned using the DNASTAR Lasergene® Megalign™ (2007) software and were manually screened for the mutation at the relevant location in the amplicon.

**Table 2.1** Components of the PCR reactions for evaluation of *kdr*

| **Reagent** | **Volume for a single reaction (µl)** |
|---|---|
| 10x PCR Buffer | 2.5 |
| dNTPs (2.5mM) | 2.5 |
| $MgCl_2$ (25mM) | 1.5 |
| AGD1 (10µM) | 3.0 |
| AGD2 (10µM) | 3.0 |
| Template | 0.5* |
| Nuclease free water | 10.3 |
| Taq DNA polymerase | 0.2 |
| **Total** | **23.5** |

* Taken from the supernatant of each tube following incubation steps of the extraction process (Section 2.2.2.1), using caution not to withdraw any parts of the mosquito leg.

### 2.2.3 World Health Organization (WHO) insecticide susceptibility assays

The insecticide resistance status of all four strains was evaluated against a range of insecticides including DDT (4.0%), permethrin (0.75%), deltamethrin (0.05%), bendiocarb (0.1%), propoxur (0.1%) and fenitrothion (1.0%). Assays were performed according to standard WHO procedures (WHO, 1998). Insecticide treated papers and exposure tubes were obtained from the WHO. Briefly, 25 female mosquitoes were placed in a holding tube for one hour and were then transferred to exposure tubes containing an insecticide treated paper for an hour before being returned to the holding tube (Figure 2.1). A 10% sugar solution was provided and mortality was recorded after 24 hours. The assays were repeated between four to five times for each strain, and for each insecticide. In each experiment a control tube was included. Mortality in the control groups was less than 5% so correction by Abbott's formula was not necessary (WHO, 1998).

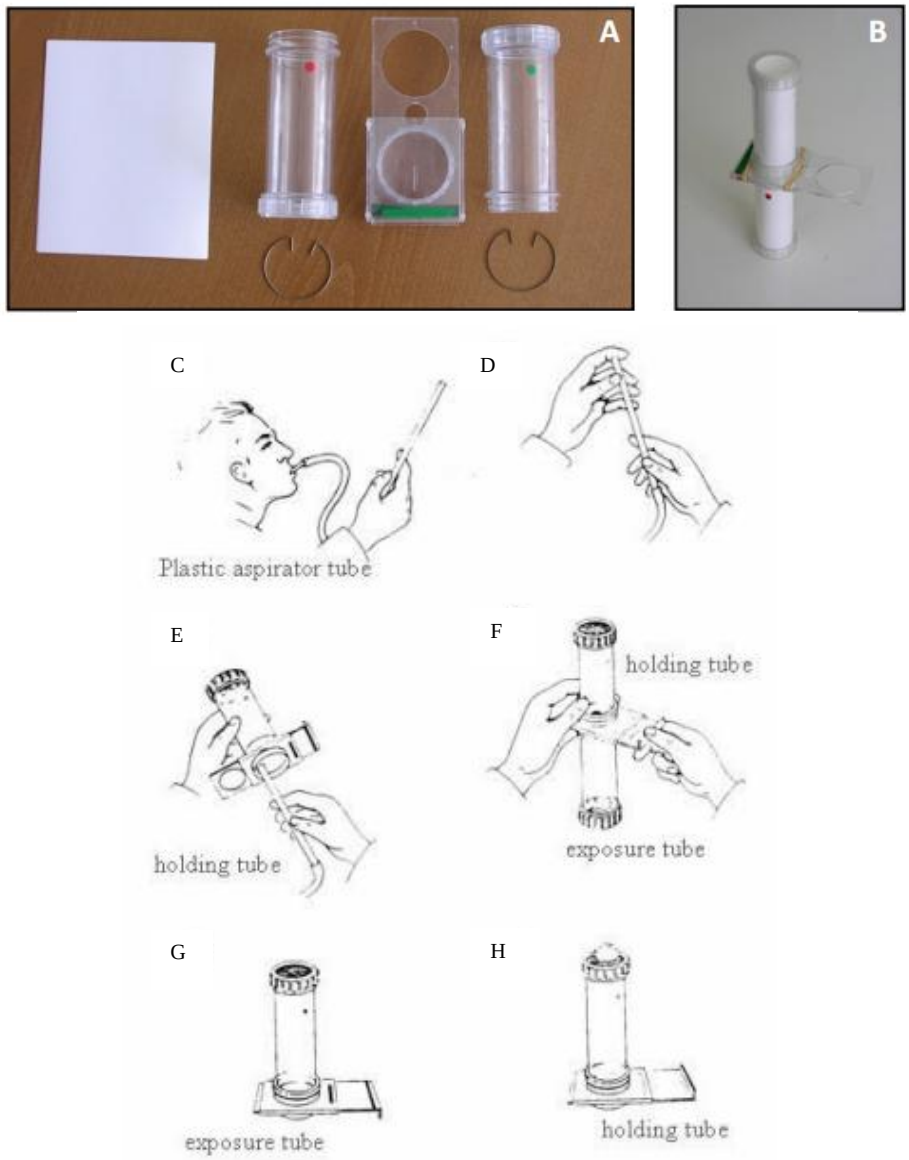



**Figure 2.1** Outline of the WHO bioassay procedure (WHO, 2006; IRAC, 2011). (A) Disassembled WHO test kit, the green markings indicate the holding portion of the system, and the red dot indicates the insecticide portion of the system. (B) An assembled test kit. (C-E) Approximately 25 mosquitoes are placed in a holding tube using an aspirator, and allowed to rest for 1 hour. (F) After the rest period, the exposure tube containing insecticide-treated paper is attached to the holding tube and the mosquitoes are transferred to the exposure tube via the sliding partition. (G-H) Mosquitoes are exposed for an hour, and then transferred back to the holding tube. Mortality is recorded after 24 hours. (H) Mosquitoes are provided with cotton wool soaked in a 10% sugar solution during the holding period.

### 2.2.4 Synergist assays

Piperonyl butoxide (PBO), an inhibitor of monooxygenase activity, and diethyl maleate (DEM), an inhibitor of GSTs, were used to synergise the resistant strains, SENN-DDT and MBN-DDT (see Appendix B for preparation of synergist papers). Twenty-five 2 to 3 day old mosquitoes were exposed to 4% PBO (SENN-DDT and MBN-DDT) or 8% DEM (MBN-DDT) for an hour, and then immediately exposed to the respective insecticide (0.05% permethrin, 0.75% deltamethrin or 4.0% DDT) for an hour before being returned to a holding tube. In addition, 25 mosquitoes were exposed to the insecticide only (0.05% deltamethrin, 0.75% permethrin or 4.0% DDT) for an hour, and then as an additional control, to the synergist only (PBO or DEM) for an hour. Mortality was recorded after 24 hours. Insecticide exposure versus synergist plus insecticide exposure were analysed using Students's *t*-test. Three to four repeats were prepared for each insecticide/synergist assay, depending on mosquito availability.

### 2.2.5 RNA extractions and preparation of amplified mRNA for microarrays

Female mosquitoes from all four strains were collected on the day of emergence and maintained on 10% sugar water. Three days later, RNA was extracted from 15 mosquitoes from each strain, representing one biological repeat. A total of three biological repeats per strain were used in the experiments and analysis described below. RNA was extracted using the PicoPure™ RNA Isolation Kit (Arcturus). Mosquitoes were thoroughly homogenised in 100µl Extraction Buffer and incubated at 42ºC for 30 minutes. The samples were centrifuged at 13 000 x *g* for two minutes, the supernatant removed and placed in a fresh microcentrifuge

tube. The supernatant was mixed with 100µl 70% ethanol before being loaded onto the extraction column. From this point, the supplied methodology was followed. This included a DNase treatment (RNase-Free DNase Set, Qiagen), as described in the PicoPure™ RNA Isolation Kit User Guide. Once the extraction was complete, the RNA concentration and quality were evaluated using a NanoDrop spectrophotometer (Thermo Scientific). RNA was used for downstream applications provided the A260/280 ratio was above 1.8, and the A260/230 ratio was close to 2. In addition, RNA quality was evaluated using native gel electrophoresis (see section 2.2.6). RNA was stored at -70°C until required.

Amplified messenger (m) RNA was prepared using the RiboAmp™ RNA Amplification Kit (Arcturus), according to supplier instructions. The kit uses a five-step process to produce high yields of the mRNA component of the total cellular RNA. The steps were as follows: a first strand synthesis reaction to produce cDNA, second strand synthesis to produce double stranded cDNA, followed by purification of cDNA, and *in vitro* transcription to produce antisense (a) RNA which was then purified. The aRNA was measured using a NanoDrop spectrophotometer. The same NanoDrop criteria as above were applied to the aRNA.

### 2.2.6 Native agarose gel electrophoresis of RNA

The protocol for native agarose gel electrophoresis of RNA was modified from Masek et al. (2005). The electrophoresis tank, gel tray, and comb and all other equipment were rinsed and sprayed with RNaseZap™ (Sigma-Aldrich). After 5 minutes all items were rinsed well with DEPC water. The running buffer (0.5x TBE, see Appendix B for preparation) and a 1% agarose gel (containing ethidium bromide [Sigma-Aldrich]) were prepared using DEPC water

(see Appendix B for preparation of DEPC water). Between 500ng - 1µg RNA was electrophoresed for each sample. Equivalent volumes of 2x RNA Loading Dye (Fermentas) were added to each sample. The loading dye contains, amongst other things, formamide which denatures the RNA so that it can separate into the smaller and larger components (18S and 28S). In addition, formamide stabilises RNA. The mixture was heated with loading dye at 70°C for 10 minutes prior to electrophoresis. Samples were electrophoresed at 70V for 30 minutes (longer electrophoresis times and voltage can damage the RNA). Single clear bands for each sample (this is known to occur in insect RNA [Winnebeck et al., 2010]) with no or minimal smearing were acceptable.

### 2.2.7 Microarrays

Preparation of probes and microarrays was based on that of Christian et al. (2011). Three independent biological repeats were performed for each strain, and for each biological repeat, two technical repeats were performed that included dye swaps in order to compensate for dye bias. Amplified antisense (a) RNA was labeled by reverse transcription using Cy-dUTPs (GE Healthcare). aRNA (8µg) was mixed with random hexamers (Invitrogen), 2µl spike in control (Lucidea Universal ScoreCard, Amersham) and water and the mixture was incubated at 70°C for 5 minutes. The reverse transcription mix (RT Buffer 5x [Invitrogen], 0.1M DTT [Invitrogen], Cy3-dUTP or Cy5-dUTP [GE Healthcare], dT-NTP mix [Invitrogen], RNasin® Ribonuclease Inhibitor [Promega] and Superscript® III [Invitrogen]) was added to each RNA and primer mix, and incubated at 50°C for 2.5 hours. The reaction was stopped by adding 1M NaOH/20mM EDTA, and incubation at 70°C for 5 minutes. The Cy-labeled cDNAs were

purified using the CyScribe™ GFX™ Purification Kit (Amersham) according to the manufacturer's instructions. In order to control the efficiency of the labeling and purification procedures, samples were measured using the microarray setting on a NanoDrop spectrophotometer. Acceptable dye binding was considered to be >0.1 pmol/µl and acceptable cDNA yields were required to be >15 ng/µl (Liverpool School of Tropical Medicine, personal communication). If these conditions were not met, the hybridisation process was abandoned. Poly(dA) (Sigma-Aldrich) was added to each cDNA mix and samples were evaporated at 37°C for an hour using an Eppendorf concentrator 5350. The cDNA was resuspended in 15.5µl hybridisation buffer (Corning) and kept in the dark until slides were ready.

During this time, the microarrays (*An. gambiae* detox chip) were prepared for hybridisation. The Pronto!™ Universal Microarray Hybridization Kit (Corning) was used, but a 1.5x preparation of each wash solution was used, along with slightly reduced exposure times, following a series of optimisation experiments. Once slides were prepared, the labeled targets were denatured by incubation at 95°C for 5 minutes. The targets were added to each array and hybridisations were performed at 42°C for 18-20 hours. After incubation, slides were washed using the Pronto!™ Universal Hybridization Kit (1.5x solutions prepared), and dried by centrifugation at 2 500 x *g* for 2 minutes.

### 2.2.8 Microarray scanning and data analysis

The arrays were scanned using the GenePix 4000B scanner (Molecular Devices, USA) where the photomultiplier tube (PMT) settings were adjusted to give a pixel ratio of approximately 1.

Spot quality and background intensities were examined and corrected using GenePix Pro 6.0 software (Axon Instruments, USA). Saturated features (occur when the pixels exceed the scanner's upper limit of detection and features appear white) were recorded as such, and were excluded from analysis.

Gene expression data were analysed using Limma version 2.12.0 (Bioconductor) (Smyth, 2005) in R, version 2.8.0 (http://cran.r-project.org/bin/windows/base/old/2.8.0/), a command-driven program for statistical computing. Raw intensity values for each spot were calculated, and then background corrected by the method "normexp" with an offset of 50 (Ritchie et al., 2007). This approach produces positive adjusted intensities and variation in log-ratios for low intensity spots are pushed toward zero (i.e. no spots are "lost" if a high background signal is measured). The corrected intensity values were transformed to log-ratios and then normalised. Control spots were used for within array normalisation (i.e. normalisation was based on non-differentially expressed control spots) (Smyth and Speed, 2003). Between array normalisation was done using the "Aquantile" method where spot intensity values are transformed so that their distributions are similar between microarrays (Smyth and Speed, 2003). MA-plots were viewed so that normalisation could be monitored. Once analyses are complete, Limma produces a "topTable", a summary that includes the following: the gene ID, M ($\log_2$-fold change) and A ($\log_2$-average intensity) values, a moderated *t*-statistic, a *p*-value, an adjusted (adj.) *p*- value, a *B*-statistic as well as an *F*-statistic (from the 'eBayes' function) (Smyth, 2004). The transcription of genes with adj. *p*-values $\leq 0.05$ and fold changes $\geq 1.5$ were considered to be statistically significant. The adj. *p*-value takes into account the fact that multiple testing is being conducted (Wright, 1992). The Benjamini and Hochberg (1995)

method, which controls the false discovery rate, was used to adjust the *p*-value. These data have been deposited into Vectorbase (https://www.vectorbase.org).

### 2.2.9 Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) was carried out in order to validate the results of the microarray experiments. As with the microarray experiment, RNA was extracted from three day old *An. arabiensis* females that had been supplied with 10% sugar solution. RNA was extracted from 15 mosquitoes from each strain (one biological repeat) using TRI® Reagent solution (Sigma-Aldrich). Briefly, 3 mosquitoes were macerated in 1ml TRI® Reagent and allowed to stand for 5 minutes at room temperature. Chloroform (0.2ml) was added to the TRI Reagent®/mosquito mixture. The tubes were gently shaken and incubated at room temperature for 5 minutes, followed by centrifugation at 12 000 x *g* (15 minutes, 4ºC). The uppermost clear aqueous layer was removed and placed in a new microcentrifuge tube. Isopropanol was added (0.5ml), and the tubes incubated for a further 5 minutes at room temperature. The RNA was precipitated by centrifugation (12 000 x *g*, 10 minutes, 4ºC), washed in 70% ethanol and once again subjected to centrifugation (7 500 x *g*, 5 minutes, 4ºC). The ethanol was removed and the samples were allowed to air dry for 5 minutes before being suspended in 30µl DEPC water. A DNase treatment was included (RNase-Free DNase Set, Qiagen), the details of which are described in Appendix B. Samples were quantified using a NanoDrop and the quality further assessed by native gel electrophoresis (section 2.2.6). Samples were then reverse transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen) according to supplier instruction.

cDNA was stored at -20°C until required for PCR. For SENN, three genes were evaluated by real-time PCR (*CYP9L1* [over-transcribed], *CO1* [saturated] and *CYP4G16* [saturated]), and for MBN, four genes were evaluated (*CYP6P3*, *CYP6AK1*, *CYP6M2* and *TPX4*, all found to be over-transcribed in the microarray study). Primers were designed based on *An. gambiae* sequence information using either Beacon Designer™ (Premier Biosoft) or Invitrogen's free online primer design tool, OligoPerfect™ Designer. For each set of strains, a reference gene evaluation was conducted and the most suitable reference gene was selected from all potential candidate genes tested (ribosomal protein *rsp 7*, ribosomal protein L19 [*RPL19*], the cytoskeletal protein *β-actin*, *GAPDH* and TATA binding protein). The data from these experiments were analysed using NormFinder (2004, Molecular Diagnostic Laboratory, Aarhus University Hospital). For the SENN strains, gene expression was measured relative to *rsp 7*, and for the MBN strains, gene expression was measured against *β-actin*. PCR was carried out using the Bio-Rad CFX96™ Real-Time PCR Detection System. Each reaction was set up using a total volume of 25µl comprising 12.5µl IQ™ SYBR super-mix (Bio-Rad), 4µl primer (concentration optimised for each gene), 1µl cDNA (100ng/µl) and nuclease free water. Primer specifics, including cycling conditions and primer concentrations are described in Table 2.2 and Table 2.3. Standard curves were prepared by two-fold dilutions of cDNA derived from the resistant strain. Three biological repeats were evaluated, and for each biological repeat, three technical repeats were included for each reaction of interest i.e. where relative quantification was calculated. Data were analysed using the Pfaffl (2001) method. Initially, the PCR product for each gene of interest was sent to Macrogen for sequencing in both directions in order to confirm (in addition to melt curve analysis) that the correct product was amplified in each case. It was necessary to clone small amplicons prior to sequencing.

**Table 2.2** SENN-base/SENN-DDT primer information for qPCR (F = forward, R = reverse, bp = base pairs)

| Gene | Primer sequence | Primer concentration | Annealing temperature | Amplicon length |
|---|---|---|---|---|
| *CYP9L1* | F 5'- AGA TAA TGT ATT CTT TCG CTA TGG -3'<br>R 5'- GCT CTT CTC GCT CTT GAA C -3' | 3.5μM | 58.3°C | 188 bp |
| *CO1* | F 5'- TGC TCC TAA AAT AGA AGA AAT TCC -3'<br>R 5'- TGC TTC CTC CTT CAT TAA CAC -3' | 3.0μM | 58.3°C | 173 bp |
| *CYP4G16* | F 5'- CAG ACC GTC CAG CCA CAT TC -3'<br>R 5'-GCG AAC GAG CAA TTA TAG GTA CTG -3' | 3.0μM | 58.3°C | 108 bp |
| *rsp 7* | F 5'-TTA CTG CTG TGT ACG ATG CC-3'<br>R 5'-GAT GGT GGT CTG CTG GTT-3' | [a] | 58.3°C | 135 bp |

[a] Primer concentration used was the same as the target gene of interest.

**Table 2.3** MBN-base/MBN-DDT primer information for qPCR (F = forward, R = reverse, bp = base pairs)

| Gene | Primer sequence | Primer concentration | Annealing temperature | Amplicon length |
|---|---|---|---|---|
| *CYP6M2* | F 5'- CAT GAC ACA AAC CGA CAA GG -3'<br>R 5'- GGT GAG GAG AGT CGA CGA AG -3' | 3.5μM | 60.0°C | 235 bp |
| *CYP6AK1* | F 5'- TCA TCG AGC GAC AGT GTA CC -3'<br>R 5'- AAA GTG TGA CCC CAG ACA GG -3' | 3.0μM | 58.3°C | 251 bp |
| *CYP6P3* | F 5'- CGA TTC TTC CTG GAC ATC GT -3'<br>R 5'- CTT GCC CAA ACT ACC GTC AT -3' | 3.0μM | 58.3°C | 141 bp |
| *TPX4* | F 5'- CAG CTG ACA GAC CGA TTA AG -3'<br>R 5'- CCG TTC GGG AAC AGT TTG TCT -3' | 3.0μM | 58.3°C | 116 bp |
| *β-actin* | F 5'- ACC AAG AGC CTG AAG CAC -3'<br>R 5'- CGA GCA CGA CAC ACT ATA TAC -3' | [a] | [b] | 123 bp |

[a] Primer concentration used was the same as the target gene of interest.

[b] Annealing temperature used was the same as the target gene of interest.

### 2.2.10 Cloning

Amplified PCR products were sent to Macrogen, but one product, *CYP4G16* (108 bp), did not produce usable sequences (it was expected that small amplicons may not be suitable for sequencing). This product was cloned so that a larger fragment was obtained, and was then re-sequenced. The cloning protocol was as follows:

#### 2.2.10.1 PCR product clean-up

qPCR product was collected from relevant reactions and purified using the QIAquick® PCR Purification Kit (Qiagen) according to supplier instructions. Sample purity and concentration were determined using a NanoDrop spectrophotometer.

#### 2.2.10.2 Ligation

The pGEM®-T Easy Vector system (Promega) was used for the ligation reactions. For each ligation reaction, a positive reaction and a negative reaction were included. Each reaction was made up to 10µl comprising 5µl 2x Rapid Ligation Buffer, 1µl pGEM®-T Easy Vector (50ng), 3µl PCR product (50ng/µl) and 1µl T4 DNA Ligase. In the case of positive controls, 2µl of "positive control" (supplied with kit) and 1µl nuclease-free water were added instead of PCR product. The reaction was mixed by pipetting and incubated overnight at 4°C. A positive ligation control produced white colonies on agar, due to the presence of an intact plasmid. The

negative control contained all the ligation reaction components, except for the PCR product. In other words, no intact plasmid was present.

#### 2.2.10.3 Transformation

Ligations were removed from the overnight 4°C incubation and were incubated at 72°C for 15 minutes. Approximately 5µl of each reaction were transferred to a cooled, sterile microcentrifuge tube. Pre-thawed (on ice) JM109 Competent Cells (Promega) were added to each ligation reaction and gently mixed. A positive control was included (provided with the kit). The reaction was heat shocked at 42°C for exactly 45 seconds and stored on ice for 2 minutes. Luria Bertani (LB) broth was added to each tube and they were then placed at 37°C for 2 hours on an orbital shaker at 200 rpm (see Appendix B for preparation of LB broth). Following incubation, 100 µl cells were spread plated onto LB agar plates containing ampicillin (100µg/µl) (see Appendix B for preparation of LB agar with ampicillin) and IPTG (0.1M)/X-gal (50mg/ml) (added using the spread plate method, before cells were spread plated). Each reaction was plated in duplicate and incubated at 37°C overnight.

#### 2.2.10.4 Colony screening

Plates were incubated at 4ºC for 2 hours in order to enhance the colour of the colonies. Positive clones were white (containing the insert of interest), while negative clones were blue. White colonies were removed using a sterile pipette tip (1 colony per tip/reaction) which in turn was used to "inoculate" a PCR reaction (Table 2.4). The universal plasmid primers, T7

(5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG-3'), were used for PCR. The PCR reaction consisted of 94°C/3 minutes, (94°C/30seconds, 56.5°C/30 seconds, 72°C/30 seconds) x 25 cycles, followed by a final extension of 72°C/10 minutes. Up to ten clones per gene were selected. Five positive ligations were also selected for PCR. In addition, colonies of interest were added to 2ml LB broth/ampicillin (50mg/ml) and were incubated at 37°C overnight. Glycerol (30%) was added to the samples which were then stored at -70°C.

Primer product was electrophoresed on a 2.5% agarose gel using 3-5µl per sample. Electrophoresis was carried out at 100 volts for 90 minutes. Fragments of the correct size (as determined using a molecular weight marker) were sent to Macrogen for sequencing.

**Table 2.4** Components of the PCR reaction required to amplify clones

| **Reagent** | **Volume for a single reaction (µl)** |
|---|---|
| 10x PCR buffer | 2.5 |
| $MgCl_2$ (25mM) | 1.5 |
| dNTPs (10mM) | 2.0 |
| SP6 primer (10µM) | 0.6 |
| T7 primer (10µM) | 0.6 |
| Clone | N/A |
| Nuclease free water | 17.55 |
| Taq DNA Polymerase | 0.25 |
| **Total** | **25** |

## 2.3 Results

### 2.3.1 WHO susceptibility testing

According to WHO criteria for assessing resistance or susceptibility (WHO, 1998), <80% mortality implies resistance, 80-97% mortality suggests that resistance be confirmed, while 98-100% mortality indicates susceptibility. These criteria, however, are being revised and it is now recommended that any survival above 2% requires further investigation (M. Coetzee, personal communication). The two base strains used in these experiments were mostly susceptible to all insecticides, except for SENN-base showing only 53.3% mortality on permethrin, and MBN-base showing 91.5% mortality on DDT (Table 2.5). The two resistant strains were both susceptible to the organophosphate, fenitrothion, while SENN-DDT was fully susceptible to one carbamate (bendiocarb) but resistant (85.5% mortality) to the other (propoxur) (Table 2.5).

**Table 2.5** Mortality data obtained following exposure of SENN-base, SENN-DDT, MBN-base and MBN-DDT to a range of insecticides, all of which belong to classes currently approved by WHO for use in vector control (*n* = number of mosquitoes exposed to insecticide).

| **Insecticide** | **SENN-base** | | **SENN-DDT** | | **MBN-base** | | **MBN-DDT** | |
|---|---|---|---|---|---|---|---|---|
| | *n* | *% mortality* | *n* | *% mortality* | *n* | *% mortality* | *n* | *% mortality* |
| DDT (4%) | 100 | 100 | 99 | 7.8 | 88 | 91.5 | 96 | 0 |
| Permethrin (0.75%) | 112 | 53.3 | 99 | 7.0 | 89 | 97.8 | 93 | 4 |
| Deltamethrin (0.05%) | 106 | 99.0 | 94 | 50.5 | 92 | 100 | 103 | 34 |
| Bendiocarb (0.1%) | 107 | 97.8 | 97 | 100 | 95 | 95.8 | 102 | 77.5 |
| Propoxur (0.1%) | 89 | 100 | 112 | 85.5 | 77 | 100 | 95 | 65.3 |
| Fenitrothion (1.0%) | 105 | 100 | 106 | 100 | 94 | 100 | 71 | 100 |

### 2.3.2 Knockdown resistance (*kdr*) detection

The AGD1 and AGD2 primers amplified a region of segment six in domain II of the sodium channel gene and the PCR product was then electrophoresed on 2.5% agarose gel in order to confirm the correct amplicon size prior to sequencing (Figure 2.2). All sequenced samples of SENN-DDT and MBN-DDT were analysed and were homozygous for the L1014F mutation. No *kdr* mutations were present in SENN-base (Oliver and Brooke, *submitted*), but in MBN-

base, 7% of the tested mosquitoes were heterozygous for the L1014F mutation with allelic frequencies of 0.04 for the resistant allele, and 0.96 for the susceptible allele.

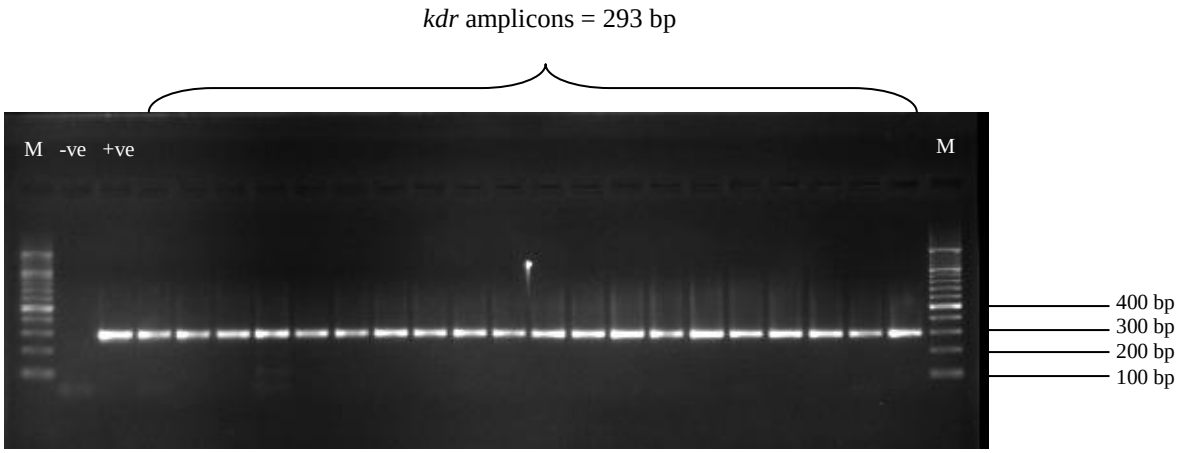



**Figure 2.2** Amplification of the S6 region, domain II of the sodium channel (SENN-DDT samples) in which the *kdr* mutation occurs. Samples were electrophoresed on a 2.5% agarose gel containing ethidium bromide; M = molecular weight marker, -ve = negative control (PCR), +ve = positive control.

### 2.3.3 Evaluation of RNA integrity by native agarose gel electrophoresis

Agarose gel electrophoresis is a commonly used method for assessing the integrity of RNA (Winnebeck et al., 2010). Typically, denatured RNA separates into the 28S and 18S rRNAs, and this can be viewed on an agarose gel as two clear bands, with minimal or no smearing. However, insect 28S rRNA frequently produces two fragments of similar size, and which are similar in size to the 18S rRNA (Winnebeck et al., 2010). These are viewed on an agarose gel as a single clear band (Figure 2.3).

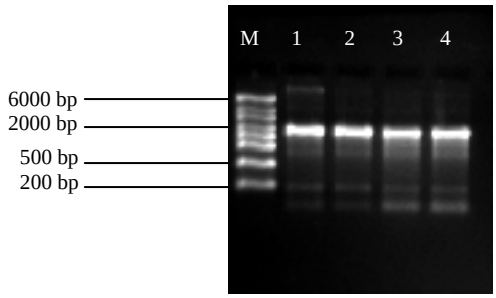



**Figure 2.3** Electrophoresis of RNA samples on a 1% agarose gel. M = RiboRuler™ High Range RNA Ladder (Fermentas), lanes 1 and 2 = RNA extracted using the PicoPure™ RNA Isolation Kit, lanes 3 and 4 = RNA extracted using the TRI® Reagent. In the case of insect RNA, denaturation frequently produces fragments of similar size (as insect RNA often contains a hidden break) that migrate closely and cannot be distinguished, as opposed to the two bands that are typically observed in electrophoresis of eukaryote RNA.

### 2.3.4 Microarrays

The *An. gambiae* detox microarray was used in a cross-species hybridisation study with *An. arabiensis*. As a result, a subset of arrays used for analysis were assessed for probe binding success (this was a visual assessment) and where probes did not hybridise, the probe name, and its position on the array were recorded. On average, 97.5% binding success rate was obtained in this study. Müller et al. (2007b) recorded a range of probe binding between 69% and 93%. The lower stringency hybridisation conditions used in this study could explain the difference observed between the two studies.

Genes that produced a fold change (FC) of ≥ 1.5 and an adjusted (adj.) *p*-value of ≤ 0.05 after microarray analysis, were considered to be differentially regulated. When SENN-DDT was compared with the relevant base strain, only one gene, *CYP9L1*, was found to be significantly over-transcribed (Figure 2.4). In SENN-base, a single gene, *CYP6Z1*, was over-transcribed. In contrast, the MBN-DDT strain had 20 genes that were significantly over-transcribed (Figure 2.5). Of these, the majority were P450 genes (50%), followed by GSTs (40%) and two redox genes (one TPX and one SOD) (Table 2.6). Five genes consistently produced saturation on both SENN and MBN microarrays. These were *CYP4G16*, *CO1*, *GSTD5*, *SOD3A* and *AGM1*. The transcription of two of these genes were investigated further by real-time PCR. These genes were validated using the SENN strains.

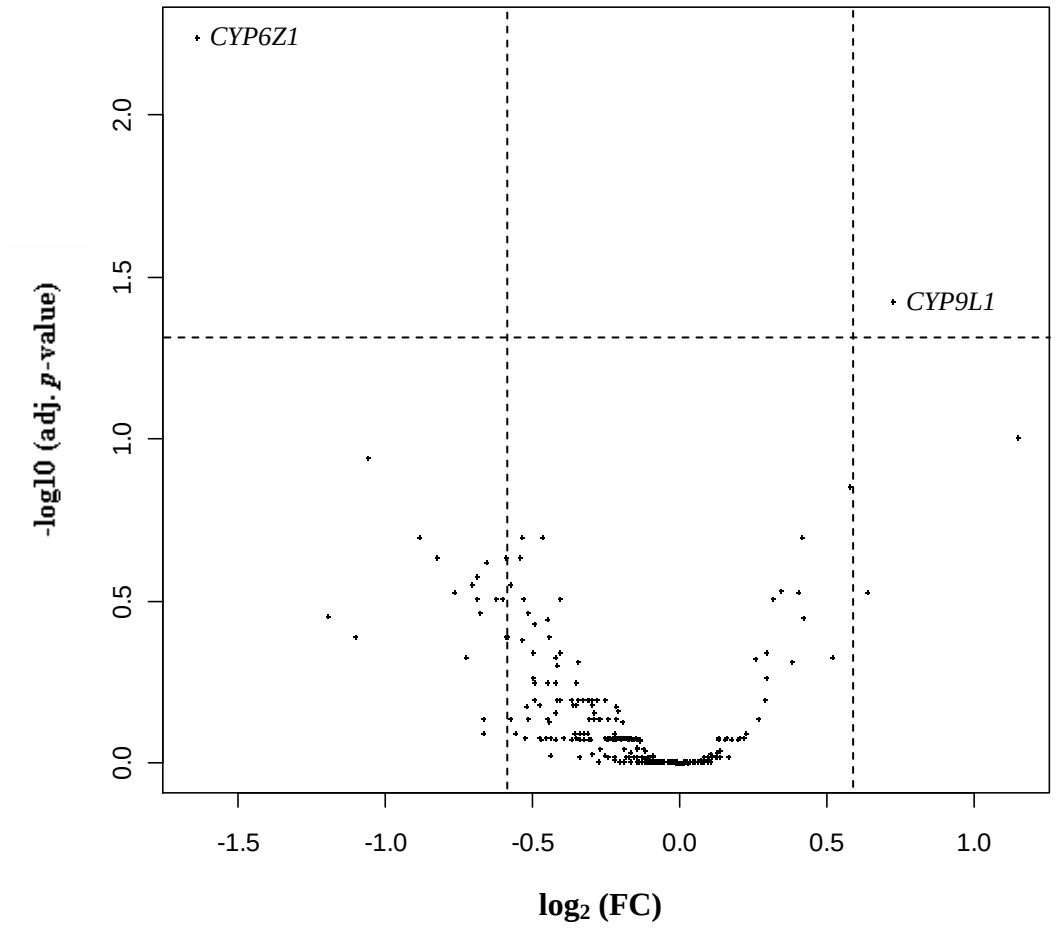



**Figure 2.4** The volcano plot of SENN-base and SENN-DDT microarray data. The plot represents both statistical relevance, in the form of the adjusted (adj.) *p*-value on the *y*-axis, and biological relevance in the form of the fold change (FC) on the *x*-axis. The cut-offs for significance are indicated by the dotted lines (adj. *p*-value ≤ 0.05; FC ≥ 1.5) and those genes that meet the criteria are labeled. Note that all positive FC values represent genes that are over-transcribed in the resistant strain (SENN-DDT), while negative FC values represent the genes that are over transcribed in the susceptible strain (SENN-base).

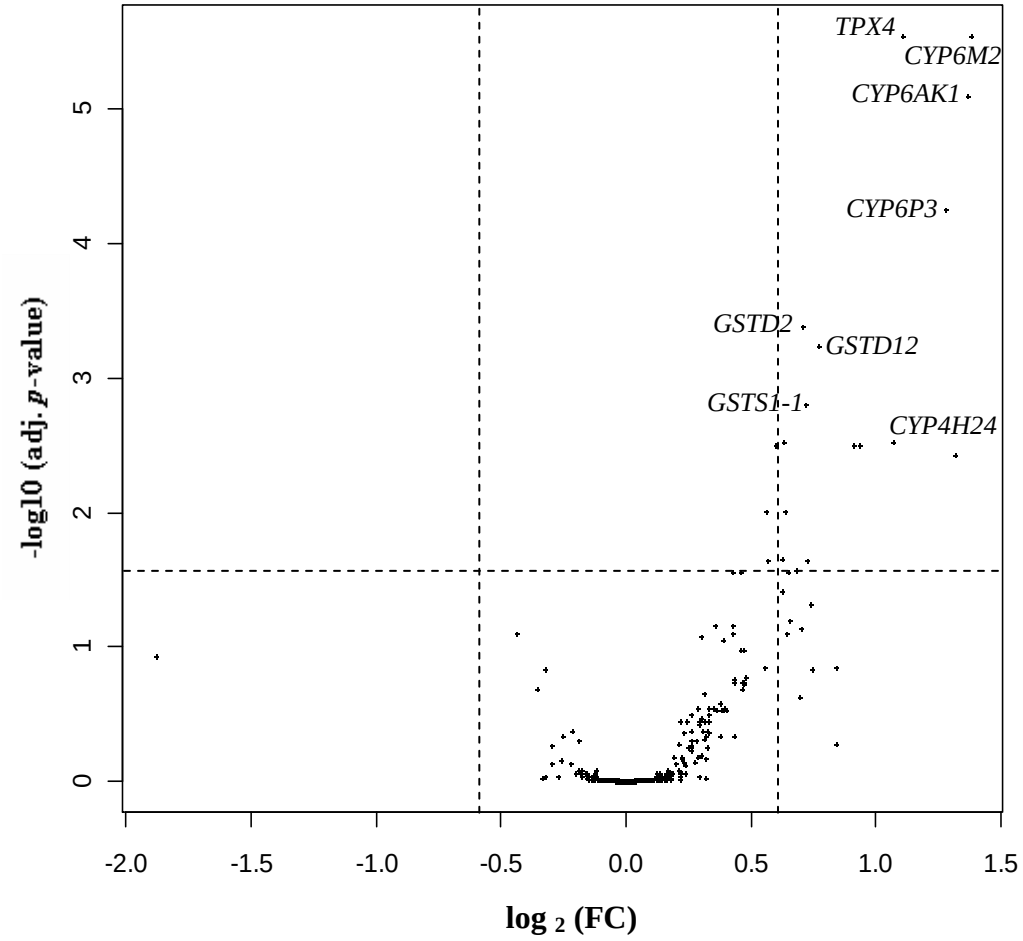



**Figure 2.5** The volcano plot of MBN-base and MBN-DDT microarray data. The plot represents both statistical relevance, in the form of the adjusted (adj.) *p*-value on the *y*-axis, and biological relevance in the form of the fold change (FC) on the *x*-axis. The cut-offs for significance are indicated by the dotted lines (adj. *p*-value ≤ 0.05; FC ≥ 1.5) and the top eight genes that met the criteria have been labeled. Note that all positive FC values belong to the genes that are over-transcribed in the resistant strain (MBN-DDT), while negative FC values represent those of the susceptible strain (MBN-base).

**Table 2.6** List of probes that were over-transcribed in SENN-DDT and MBN-DDT when compared with the susceptible equivalents. Relevant information included is the gene function, fold change (FC), adj. *p*-value, Genbank (GB) accession number and the chromosomal location of each gene in the *An. gambiae* genome. E = exponent.

| Gene | Function | FC | Adj. *p*-value | GB accession number | Location |
|---|---|---|---|---|---|
| **SENN** | | | | | |
| *CYP9L1* | Cytochrome P450 | 1.7 | 3,74E-2 | AF487781 | 3L |
| | | | | | |
| **MBN** | | | | | |
| *CYP6M2** | Cytochrome P450 monooxygenase | 2.7 | 6.12E-6 | AY193729 | 3R |
| *TPX4** | Thioredoxin-dependent peroxidase | 2.3 | 6.12E-6 | AY745235 | 3L |
| *CYP6AK1** | Cytochrome P450 monooxygenase | 2.6 | 2.12E-5 | AY745227 | 3L |
| *CYP6P3** | Cytochrome P450 monooxygenase | 2.6 | 1.20E-4 | AF487534 | 2R |
| *GSTD2* [#] | Glutathione S-transferase | 1.7 | 3.09E-4 | Z71480 | 2R |
| *GSTS1-1** | Glutathione S-transferase | 1.7 | 7.49E-4 | L07880 | 3L |
| *GSTD12* [#] | Glutathione S-transferase | 1.7 | 1.44E-3 | AF316638 | 2R |
| *CYP4H24** | Cytochrome P450 monooxygenase | 2.2 | 4.83E-3 | AY062206 | X |
| *GSTD3* [#] | Glutathione S-transferase | 2.0 | 4.91E-3 | AF513638 | 2R |
| *CYP6AG2** | Cytochrome P450 monooxygenase | 2.0 | 5.24E-3 | AY745224 | 2R |
| *GSTMS3* [#] | Glutathione S-transferase | 1.6 | 5.83E-3 | AY278448 | 3R |
| *GSTS1-2** | Glutathione S-transferase | 1.5 | 6.71E-3 | AF513639 | 3L |
| *CYP9J5** | Cytochrome P450 monooxygenase | 2.7 | 7.73E-3 | AY748830 | 3L |
| *CYP6P1** | Cytochrome P450 monooxygenase | 1.5 | 7.73E-3 | AY028785 | 2R |
| *SOD1** | Superoxide dismutase | 1.6 | 1.13E-2 | AY505417 | 3L |
| *CYP6M3** | Cytochrome P450 monooxygenase | 1.8 | 1.58E-2 | AY193730 | 3R |
| *GSTU1** | Glutathione S-transferase | 1.6 | 1.58E-2 | AF515521 | X |
| *CYP12F2** | Cytochrome P450 monooxygenase | 1.7 | 1.83E-2 | AY176050 | 3R |
| *GSTMS1* [#] | Glutathione S-transferase | 1.6 | 3.81E-2 | AY278446 | X |
| *CYP12F4** | Cytochrome P450 monooxygenase | 1.7 | 4.01E-2 | AY176048 | 3R |

[#] These genes are unique to the DDT-selected strain, MBN-DDT, resistant to DDT, permethrin and deltamethrin

* These genes are shared with the permethrin resistant strain, KWAG-perm (Chapter 3, Table 3.2)

### 2.3.5 qPCR

#### 2.3.5.1 Confirmation of qPCR product

All qPCR products were sent to Macrogen Europe for sequencing, and where amplicons were large enough (i.e. > 110 bp), sequencing was successful. These sequences were inserted into a BLAST search using NCBI and the correct product confirmed. The product of *CYP4G16*, was unsuccessfully sequenced due to its small size (108 bp). As a result, the amplicon was cloned into the pGEM®-T Easy plasmid system. Clones were visualised by electrophoresis on a 2.5% agarose gel (Figure 2.6) in order to determine if the correct product had been cloned. The *CYP4G16* amplicon was 108 bp and with the primer sequences, SP6 (100 bp) and T7 (78 bp), the clone size was 286 bp. Once the sequences of clones were returned, the correct product was confirmed in a BLAST search.

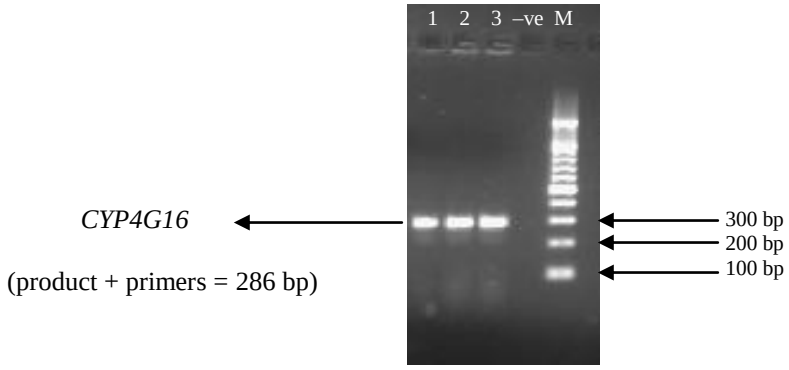



**Figure 2.6** Amplification of cloned *CYP4G16*. Samples were electrophoresed on a 2.5% agarose gel containing ethidium bromide; M = molecular weight marker, -ve = negative PCR control.

### 2.3.5.2 Reference gene selection and microarray validation

Relative quantification was used to validate the microarray data. Reference gene analysis was conducted prior to the experiments in order to evaluate which reference genes showed the most stable expression in each strain (SENN gene expression was measured relative to *rsp 7*; MBN gene expression was measured relative to *β-actin*). Tables 2.7 and 2.8 show the output format of NormFinder, the software used for reference gene analysis. The expression level of *CYP9L1* in the SENN-DDT strain had a fold change (FC) of 1.7 after microarray analysis, and FC of 2.5 after qPCR analysis (Figure 2.7). While saturated spots were flagged and not used in analyses, qPCR was used on two of them to measure the FC difference between the susceptible and resistant SENN strains. Results showed that the cytochrome P450, *CYP4G16*, produced an FC of 1.8, while *CO1*, a gene frequently associated with the resistant phenotype, produced an FC of 1.6 (Figure 2.7).

In the case of the South African *An. arabiensis* (MBN), a sample of four genes that were over-transcribed according to microarray evaluation were further validated. These were the four top genes based on FC and the adj. *p*-value namely *CYP6M2, TPX4, CYP6AK1* and *CYP6P3* (Figure 2.8). Except for *CYP6AK1*, the FCs in expression after microarray analyses were comparable to those measured by qPCR. Based on qPCR analysis, *CYP6M2* and *CYP6P3* each had fold change expression levels of more than 2 while *CYP6AK1* and *TPX4* produced fold change values of 0.9 and 1.5 respectively (Figure 2.8).

**Table 2.7** The output provided by NormFinder in Excel following reference gene analysis for the SENN strains. The software provides a stability value for each reference gene tested. The gene with the lowest stability value is the top ranked gene i.e. the reference gene with the most stable expression.

| **Gene name** | **Stability value** | | **Best gene** | *rsp 7* |
|---|---|---|---|---|
| *β-actin* | 0.349 | | **Stability value** | 0.121 |
| *TBP* | 0.279 | | | |
| *RPL19* | 0.218 | | **Best combination of two genes** | *rsp 7* and *GAPDH* |
| *GAPDH* | 0.138 | | **Stability value for best combination of two genes** | 0.102 |
| *rsp 7* | 0.121 | | | |

**Table 2.8** The output provided by NormFinder in Excel following reference gene analysis for the MBN strains. The software provides a stability value for each reference gene tested. The gene with the lowest stability value is the top ranked gene i.e. the reference gene with the most stable expression.

| **Gene name** | **Stability value** | | **Best gene** | *β-actin* |
|---|---|---|---|---|
| *TBP* | 0.390 | | **Stability value** | 0.042 |
| *rsp 7* | 0.243 | | | |
| *RPL19* | 0.167 | | **Best combination of two genes** | *β-actin* and *GAPDH* |
| *GAPDH* | 0.130 | | **Stability value for best combination of two genes** | 0.100 |
| *β-actin* | 0.042 | | | |

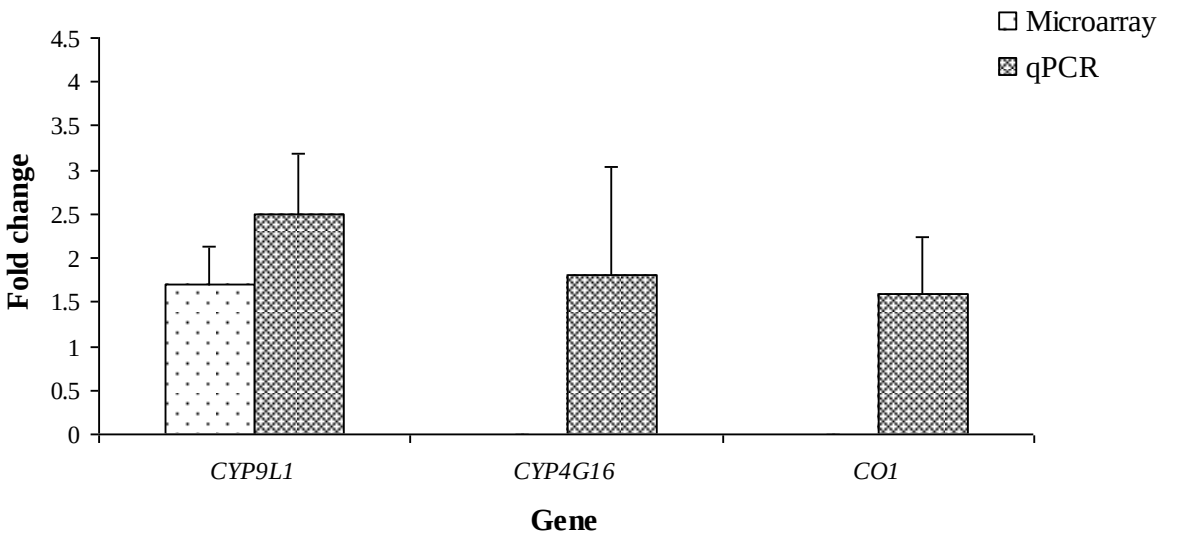



**Figure 2.7** A comparison of the outcome of gene expression evaluation (mean±SD) by microarrays and by qPCR in selected genes in SENN. Genes of interest were measured against the relevant reference gene (*rsp 7*). The genes *CYP4G16* and *CO1* were saturated on the microarrays, and therefore no microarray FC values for these are present.

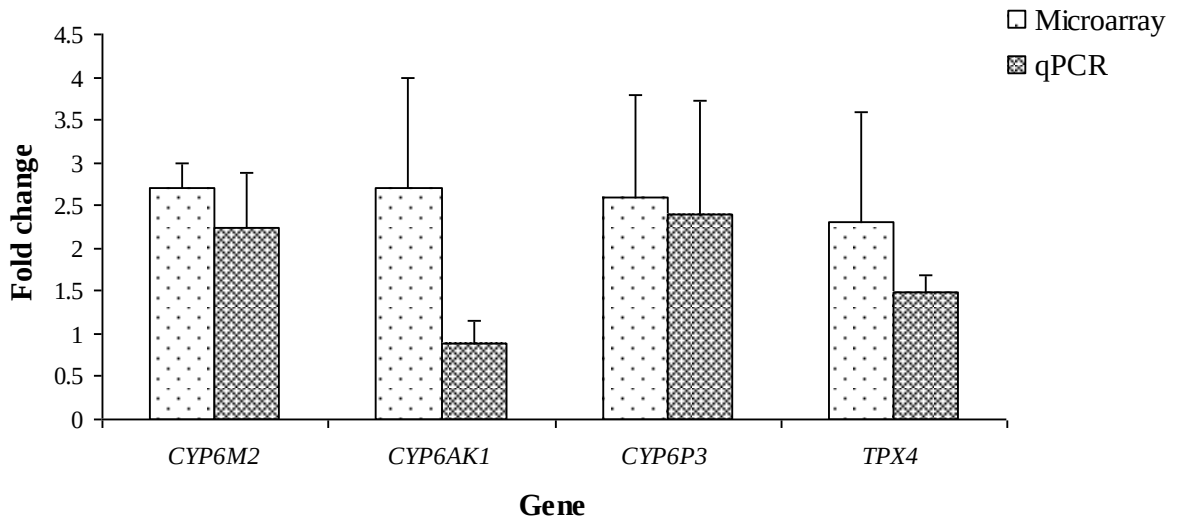



**Figure 2.8** A comparison of the outcome of gene expression evaluation (mean±SD) by microarrays and by qPCR in selected genes in MBN. Genes of interest were measured against the relevant reference gene (*β-actin*).

### 2.3.6 Synergist assays

The synergist assays were used to determine whether the expression of detoxification genes in each resistant strain were in fact related to the resistance observed, or whether the phenotypes were due to the presence of *kdr*. As only one gene was over-transcribed in SENN-DDT (a cytochrome P450) only PBO was used as a synergist for this strain. No significant differences in mortality were observed between DDT exposure versus exposure to PBO + DDT or permethrin versus PBO + permethrin (Table 2.9). However, the mortality on deltamethrin versus PBO + deltamethrin was significantly different ($p = 0.0006$, $t = 7.7308$, df = 5) (Table 2.9). The effects of both DEM and PBO were evaluated in the MBN-DDT strain as monooxygenases and GSTs were over-transcribed in the resistant phenotype according to the microarray experiments. The synergist, PBO, had no significant impact on mosquito response to DDT or permethrin but, as for SENN-DDT, did impact significantly on MBN-DDT response to deltamethrin ($p = 0.0004$, $t = 8.331$, df = 5). While DEM had no significant impact on DDT resistance, a significant difference on mosquito response to deltamethrin versus deltamethrin + DEM synergist ($p = 0.0083$, $t = 4.8596$, df = 4) was observed (Table 2.9).

**Table 2.9** Percentage mortality of SENN-DDT and MBN-DDT mosquitoes (females and males) to DDT, permethrin and deltamethrin following exposure to synergists (*n* = number of mosquitoes exposed).

| Colony | Treatment | *n* | % Mortality (± SD) |
|---|---|---|---|
| **SENN-DDT** | PBO (4%) + DDT (4%) | 107 | 3.9 (± 4.7) |
| | DDT (4%) only | 107 | 13.0 (± 8.6) |
| | PBO (4%) only | 80 | 0 |
| | PBO (4%) + deltamethrin (0.05%) | 126 | 83.8 (± 1.3)* |
| | Deltamethrin (0.05%) only | 89 | 25.3 (± 15.6)* |
| | PBO (4%) only | 80 | 0 |
| | PBO (4%) + permethrin (0.75%) | 75 | 0 |
| | Permethrin (0.75%) only | 72 | 1.3 (± 2.3) |
| | PBO (4%) only | 79 | 2.7 (± 4.6) |
| **MBN-DDT** | PBO (4%) + DDT (4%) | 79 | 2.3 (± 2.1) |
| | DDT (4%) only | 71 | 1.3 (± 2.3) |
| | PBO (4%) only | 81 | 1.1 (± 2.0) |
| | PBO (4%) + deltamethrin (0.05%) | 78 | 70.3 (± 16.5)* |
| | Deltamethrin (0.05%) only | 97 | 2.2 (± 4.3)* |
| | PBO (4%) only | 81 | 1.1 (± 2.0) |
| | PBO (4%) + permethrin (0.75%) | 74 | 1.3 (± 2.3) |
| | Permethrin (0.75%) only | 73 | 6.7 (± 4.6) |
| | PBO (4%) only | 74 | 1.3 (± 2.3) |
| **MBN-DDT** | DEM (7%) + DDT (4%) | 80 | 1.5 (± 2.6) |
| | DDT (4%) only | 72 | 4.0 (± 4.0) |
| | DEM (7%) only | 82 | 3.5 (± 3.7) |
| | DEM (7%) + deltamethrin (0.05%) | 74 | 46.0 (± 1.7)* |
| | Deltamethrin (0.05%) only | 78 | 16.8 (± 10.2)* |
| | DEM (7%) only | 82 | 3.5 (± 3.7) |
| | DEM (7%) + permethrin (0.75%) | 75 | 1.7 (± 2.9) |
| | Permethrin (0.75%) only | 75 | 0 |
| | DEM (7%) only | 69 | 3.0 (± 2.7) |

* Indicates significant differences between insecticide versus synergist plus insecticide.

## 2.4 Discussion

Resistance to DDT and pyrethroids is widespread and has hampered malaria control efforts throughout Africa (Hemingway, 1983; Hargreaves et al., 2003; Casimiro et al., 2006; Abdalla et al., 2008; Mouatcho et al., 2009; Balkew et al., 2010; Matowo et al., 2010; Yewhalaw et al., 2011). Artificial selection in the laboratory allows one to mimic the development of insecticide resistance from repeated and continuous exposure to insecticides, a situation that wild vector populations are frequently exposed to.

The two resistant *An. arabiensis* strains that were used in this study (one from South Africa and the other from Sudan) have been under DDT selection pressure for many years in the laboratory. Bioassay data confirmed that both SENN-DDT and MBN-DDT are highly resistant to DDT. In addition, the two strains were found to be resistant to pyrethroids (deltamethrin and permethrin). The South African population showed additional resistance to both carbamates tested while low-level resistance to propoxur was recorded in the Sudanese strain. SENN-base was resistant to permethrin, and MBN-base showed low-level resistance to DDT. The outcomes of the synergist experiment suggest that resistance to these insecticides in the base strains would not have skewed the microarray data (as metabolic detoxification appears to be largely responsible for deltamethrin resistance).

The development of multiple insecticide resistance in the above mentioned strains is supported by earlier studies published on the same laboratory populations. The MBN strain was colonised in 2002 without detecting pyrethroid resistance in the population. However, three years later Mouatcho et al. (2009) reported the presence of pyrethroid resistance which was

rapidly selected for (within four generations) in the laboratory and was shown to be P450 based. The same authors also showed that carbamate tolerance could be selected for from the same strain. Ranson et al (2009) recently published a country wide study and showed that *An. arabiensis* populations from Sudan are resistant to both DDT and pyrethroids, but remained fully susceptible to carbamates and the organophosphate, fenitrothion. The data presented here support what has been observed in earlier studies.

The fact that DDT and pyrethroid resistance in *An. gambiae* are linked has been well-documented and has been attributed to the presence of *kdr* mutations (Martinez-Torres et al., 1998; Ranson et al., 2000). Specifically, *kdr* is strongly linked with DDT and permethrin resistance, and less so with deltamethrin resistance (Ramphul et al., 2009; Brooke and Koekemoer, 2010). In *An. arabiensis*, the relationship between the presence of *kdr* mutations and resistance phenotype is more complicated (Matambo et al., 2007; Balkew et al., 2010). For example, Matambo et al. (2007) reported the presence of the L1014F mutation in the resistant SENN strain but reported little correlation between the presence of the mutation, and the outcome of bioassay data. In the present study, the SENN-DDT strain still shows the presence of the L1014F mutation in 100% of the population which is now correlated with high resistance to DDT and permethrin but only moderate (50%) resistance to deltamethrin. The South African *An. arabiensis* population has previously been confirmed not to carry any *kdr* mutations (Hargreaves et al., 2003; Mouatcho et al., 2009). However, the continued selection pressure from exposure of MBN-DDT to DDT has resulted in this strain showing the L1014F mutation at a frequency of 100%. The L1014S mutation is absent from both laboratory strains. The presence of the resistant allele in MBN-base is likely to be responsible for the low-level resistance to DDT (91.5% mortality) that was observed in the bioassays. Different cohorts of

each strain were used for the WHO bioassays and *kdr* analysis. In future work, it would be ideal to perform *kdr* analysis on the survivors of WHO bioassays.

The detoxification enzyme profiles of the two laboratory selected DDT resistant *An. arabiensis* strains was investigated using cross-species hybridisations of *An. arabiensis* genetic material with the *An. gambiae* detoxification microarray (detox chip). Of the 98% of probes that hybridised, only one gene in the SENN-DDT strain was over-transcribed. This was the cytochrome P450, *CYP9L1*. This was in contrast to MBN where a similar success rate of probe hybridisation was recorded, but 20 genes were highly transcribed in the resistant strain.

The use of the *An. gambiae* detox chip allows for the evaluation of transcription of a large number of genes simultaneously but the criteria one uses to find significance will determine how many genes are of interest for further study. In other studies (both same- and cross-species hybridisations) the cut-off for significance in terms of fold change ranged from >1.5 to 2.0, and the *p*-value cut-off for significance ranged from < 0.001 to <0.05 (Girardot et al., 2004; Poupardin et al., 2008; Vontas et al., 2007; Christian et al., 2011). Generally, where a higher fold change (FC) was used as criteria to identify over-transcribed genes, a lower *p*-value cut-off was also used to determine significance, and vice versa. In this study, the stringency was adjusted for the wash solutions by increasing the required amount of each solution (i.e. higher than what was recommended by the supplier). These experimental conditions produced the best arrays, but because the experiment was based on cross-species hybridisations, less strict criteria were used for identifying those genes with a significant level of differential transcription.

The action of the P450-dependent monooxygenases is one of the ways in which insects become resistant to insecticides (Scott, 1999). Only one gene, *CYP9L1*, showed high expression levels in the SENN-DDT strain and is likely to play a key role in the observed resistance to deltamethrin (based on the outcomes of the synergist experiment). The CYP9 gene family is closely related to the CYP6 family (highly expressed in the MBN-DDT strain) (Rose et al., 1997) and members of this family have been linked to insecticide resistance in a number of insects (Pittendrigh et al., 1997; Rose et al., 1997; Strode et al., 2008; Zhou et al., 2010). In the susceptible strain, SENN-base, the gene *CYP6Z1* was over-transcribed when compared to SENN-DDT. However, genes over-transcribed in the susceptible strains were beyond the scope of the study.

A number of genes were consistently saturated when both MBN- and SENN-DDT arrays were analysed. Some of these were mainly saturated in one channel, and less so in the other, which raises the possibility that a gene is over-transcribed, but this is masked by the saturation, and might therefore be overlooked. Two of these, *CYP4G16* and *CO1*, were investigated further using qPCR and SENN-DDT. The monooxygenase, *CYP4G16* was chosen because it has previously been linked to pyrethroid tolerance in *An. arabiensis* (FC = 4.5) (Müller et al., 2007b). The cytochrome oxidase gene, *CO1* was selected as it was over-transcribed in a microarray study on pyrethroid resistant *An. funestus* (FC = 2.7), (Christian et al., 2011). In this study, we obtained lower FC values of 1.8 and 1.6 for *CYP4G16* and *CO1* respectively after qPCR analysis. While these values are relatively low when compared with previously reported data, their involvement, if any, in DDT resistance and the reason for saturation should be investigated further.

According to the criteria used in this study, 20 genes were differentially regulated in the MBN-DDT strain and most of these genes belong to the monooxygenase and GST enzyme groups. In addition, most of the over-transcribed CYP genes belonged to the CYP6 family which is frequently associated with insecticide resistance in insects. Based on microarray analysis, the four most significantly over-transcribed genes were *CYP6M2*, *TPX4*, *CYP6AK1* and *CYP6P3*. All four genes were selected for qPCR validation, however, significant over-transcription was confirmed in only two of these genes (*CYP6M2* and *CYP6P3*). Recently, Munhenga and Koekemoer (2011) used qPCR to assess the transcription of a range of monooxygenase genes in a pyrethroid-selected *An. arabiensis* strain from the same geographical area in South Africa. These authors found that *CYP6Z1* (FC = 4.7), *CYP6Z2* (FC = 1.7) and *CYP6M2* (FC = 2.2) were significantly over-transcribed.

It is important to note that the use of qPCR can be more reliable as a measure of gene over-transcription due to the use of gene specific primers (versus the use of the *An. gambiae* microarray in a cross-species hybridisation). The preparation of a similar *An. arabiensis* microarray would be extremely valuable and might improve detection of specific detoxification genes involved in insecticide resistance.

Of the CYP genes that were over-transcribed in the current study, according to microarray evaluation, a number have been implicated in insecticide resistance in *An. gambiae*. Djouaka et al. (2008) found that *CYP6P3* and *CYP6M2* were both upregulated in pyrethroid-resistant *An. gambiae* populations in Benin and Southern Nigeria. In permethrin resistant *An. gambiae* from Ghana, *CYPM2*, *CYP6AK1* and *CYP6P3* were amongst the top 10 differentially expressed genes in resistant mosquitoes (Müller et al., 2008).

Eight GSTs were significantly over-transcribed in the enzyme profile of the MBN-DDT strain. These enzymes are able to detoxify xenobiotics and endogenous compounds by conjugation, dehydrochlorination, peroxidase activity, or sequestration (Che-Mendoza et al., 2009). The epsilon class GSTs have been specifically linked to DDT resistance in *An. gambiae* (Brown, 1986; Ranson et al., 1997; Ranson et al., 2001; Ding et al., 2003; Che-Mendoza et al., 2009) and delta class GSTs to a lesser extent (Che-Mendoza et al., 2009). Furthermore, GSTs have more recently been linked to pyrethroid resistance in other insects (Kostaropoulos et al., 2001; Vontas et al., 2001) and so their presence in the resistance profile of MBN-DDT might be linked directly to protection against the pyrethroids. Because they help to protect cells against oxidative stress, their over-expression in MBN-DDT is also likely to be linked to the action of the cytochrome P450s where the GSTs are involved in secondary metabolism through the action of glutathione peroxidase (Che-Mendoza et al., 2009).

A number of enzymes, such as the SODs and TPXs, counteract the effects of reactive oxygen molecules which are harmful to the host (Corona and Robinson, 2006). The SODs function by converting superoxide anions to hydrogen peroxide and oxygen (Fridovich, 1978). In turn, the TPXs are involved in the removal of hydrogen peroxide (Fridovich, 1978). Based on microarray experiments, we reported high levels of *TPX4* (2.3 fold) expression in the South African MBN-DDT *An. arabiensis*. This enzyme was shown to be over-transcribed in *An. arabiensis* during the spraying season of a cotton crop in Cameroon (Müller et al., 2007b), while *TPX1* was over-expressed in *An. gambiae*, resistant to pyrethroids, from Ghana (Müller et al., 2007a). In MBN-DDT, whether the high expression of *TPX4* is related directly to the activities of the P450 enzymes (to counteract metabolic byproducts), or is a function of the

insecticide resistance selection process where they are on “stand-by” to provide protection against pyrethroids, is unknown.

According to Brooke and Koekemoer (2010), and references therein, the correlation between the presence of *kdr*, and mosquito response to insecticide is strongest in the case of DDT, less so with permethrin, and weakest with deltamethrin. The outcome of the synergist studies performed here suggests that detoxification enzymes have minimal impact on DDT and permethrin resistance in these strains, but are very important for protection against the pyrethroid, deltamethrin. However, the presence of *kdr* mutations is likely to assist in protection against DDT and permethrin. It would be valuable to assess whether higher doses or longer exposures to permethrin have any impact on the results of the synergist study (it is possible that metabolic resistance may have been observed under these circumstances).

The combination of expression data and synergist data suggests that the mechanisms conferring insecticide resistance are extremely complex. There is a lack of understanding as to how these genes interact and support each other in the detoxification of specific insecticides and further investigation into these molecular mechanisms is needed. The data suggests that the metabolic mechanisms associated with resistance in each strain are different for that population as there was no single gene that showed an increase in transcription between South Africa and Sudan. However, a number of genes identified in this study as being over-transcribed have been flagged in other studies for their possible roles in insecticide resistance of *An. arabiensis*; examples include *CYP6M2*, *CYP6Z2*, *GSTS1-2* and *TPX4.* It would be valuable to replicate this study on wild populations from these regions and compare the results with those found on laboratory strains.

# Chapter 3

## The detoxification enzymes associated with permethrin resistance in a laboratory strain of *Anopheles arabiensis* from South Africa

## 3.1 Introduction

Pyrethroids are the most commonly used insecticides: they are most often used for indoor residual spraying, and are the only class of insecticide approved for treatment of bednets due to their relatively low level of mammalian toxicity (WHO, 1989; Coosemans and Carnevalle, 1995; Liu et al., 2006). However, pyrethroid resistance in *Anopheles arabiensis* is well documented and has been reported in many African countries (Matambo et al., 2007; Müller et al., 2007b; Abdalla et al., 2008; Mouatcho et al., 2009; Balkew et al., 2010; Yewhalaw et al., 2011).

*Anopheles arabiensis* is one of the major African malaria vectors and the main vector in South Africa (Coetzee et al., 2000). Historically, this species was considered to be susceptible to all classes of insecticide. In 1996 an extensive study of insecticide susceptibility was conducted using *An. arabiensis* from three areas in South Africa (KwaZulu-Natal [KZN], Limpopo and Kruger National Park) (Gericke et al., 2002). Bioassays revealed that mosquitoes from all three areas were susceptible to DDT and deltamethrin (Gericke et al., 2002). In 2003, *An. arabiensis* sampled from two areas in KZN were found to be resistant to DDT, but susceptible to deltamethrin (Hargreaves et al., 2003). A more recent study, conducted in 2005, indicated that *An. arabiensis* in KZN were susceptible to deltamethrin, but signs of resistance to permethrin were evident (Mouatcho et al., 2009).

There are numerous reports implicating P450s in permethrin resistance, however, less is known about the specific enzymes that play a role in this type of resistance, and in the case of *An. arabiensis* less is known in this context when compared with *An. gambiae*. Genes that

have been implicated in pyrethroid resistance in *An. arabiensis* include *CYP6M2, CYP6Z1*, *CYP6Z2*, *CYP6P3*, *CYP4H24* and *TPX4* (Müller et al., 2007b; Munhenga and Koekemoer, 2011; Nardini et al., 2012). The aim of this study was to evaluate the transcript abundance of detoxification genes associated with a colonised, permethrin resistant South African population of *An. arabiensis*.

## 3.2 Materials and methods

### 3.2.1 Mosquito strains

The *An. arabiensis* permethrin resistant strain, KWAG-perm, and the equivalent susceptible strain (KWAG) were used for this experiment. KWAG originated from Mamfene in KwaZulu-Natal (KZN) and was permethrin resistant (78.05%) at the time of colonisation (Mouatcho et al., 2009). However, in the absence of selection by insecticide exposure, the strain reverted back to the susceptible state. When selection was placed on a sub-population, resistance was rapidly attained and this strain was named KWAG-perm (Mouatcho et al., 2009). Mosquitoes were reared as described in Chapter 2 (section 2.2.1).

### 3.2.2 RNA extractions and amplified mRNA synthesis for microarrays

RNA was extracted from batches of 15 female mosquitoes three days after emergence. This represented one biological repeat, and in total, RNA was extracted for three biological repeats of each strain. RNA extraction and mRNA amplification are described in Chapter 2 (section 2.2.5).

### 3.2.3 Microarrays

Three independent biological repeats were prepared for each strain, and for each biological repeat, two technical repeats were performed that included dye swaps in order to compensate for dye bias. Microarrays were prepared as described in Chapter 2 (section 2.2.7).

### 3.2.4 Microarray scanning and data analysis

The microarrays were scanned as described in Chapter 2 (section 2.2.8), but analyses of gene expression data were modified slightly due to artifacts present on this batch of the arrays and which were associated with the printing process. Gene expression data were analysed using Limma version 2.12.0 (Bioconductor) (Smyth, 2005) in R, version 2.8.0 (http://cran.r-project.org/bin/windows/ base/old/2.8.0/). Raw intensity values for each spot were calculated, and then background corrected by the method "normexp" with an offset of 50 (Ritchie et al., 2007). The corrected intensity values were transformed to log-ratios and then normalised. Composite Loess was used for within array normalisation (Smyth and Speed, 2003). In this method, control spots and features, per sub-array, are used for producing non-linear, best-fit lines (van Heerden et al., 2007). The use of control spots ensures that the resulting best-fit line is not biased by differential expression of genes. Conversely, the use of all genes for normalisation improves stability with respect to the number of spots, and most importantly, in this instance, where sub-array Loess curves were used, provides flexibility in terms of print-tip group trends that one might observe (Smyth and Speed, 2003). Print-tip peculiarities were present in some slides, hence the choice of normalisation method. The "Aquantile" method was used for between array normalisation (Smyth and Speed, 2003). Genes with adjusted

(adj.) $p$-values $\leq 0.05$ and fold changes (FC) $\geq 2.0$ were considered to be statistically significant. These data have been deposited into Vectorbase (https://www.vectorbase.org).

### 3.2.5 Quantitative real-time PCR (qPCR)

Real-time PCR was carried out in order to validate the outcome of the microarray experiments. RNA was extracted from 15 three day old *An. arabiensis* females (representing one biological repeat) that had been supplied with 10% sugar solution. TRI® Reagent solution (Sigma-Aldrich) was used according to the supplied methodology (see Chapter 2, section 2.2.9 for details).

Two genes, *CYP6AG2* and *TPX2*, were evaluated using real-time PCR. Beacon Designer™ (Premier Biosoft) or Invitrogen's free online primer design tool, OligoPerfect™ Designer were used to design primers. These were based on *An. gambiae* sequence information. The reference gene (and reference gene primers) used for qPCR was based on that of Munhenga and Koekemoer (2011) who also conducted studies on the KWAG strains. These authors reported that *18S rRNA* showed the most stable expression of the 6 potential reference genes tested. PCR was carried out as described in Chapter 2 (section 2.2.9). Primer sequences and specific annealing temperatures are shown in Table 3.1.

**Table 3.1** KWAG/KWAG-perm primer information for qPCR (F = forward, R = reverse)

| Gene | Primer sequence | Primer concentration | Annealing temperature |
|---|---|---|---|
| *CYP6AG2* | F 5'- TTG TGC TGC CGT ACT ATT CG-3'<br>R 5'- TAC TAT CGC CCG TCT CAC CT -3' | 2.0μM | 59.4°C |
| *TPX2* | F 5'- GGA TGT TTG TGG GGA ATA CG -3'<br>R 5'- TGT GCG ATT AGC CTC CTC TT-3' | 3.5μM | 56.3°C |
| *18S* | F 5'- TAC CTG GGC GTT CTA CTC -3'<br>R 5'- CTT TGA GCA CTC TAA TTT GTT C -3' | [a] | [a] |

[a] Primer concentration and annealing temperature was the same as that of *CYP6AG2* and *TPX4* when used as a reference gene in each case.

## 3.3 Results

KWAG and KWAG-perm have been evaluated for insecticide resistance on an ongoing basis in the laboratory. The base strain shows low-level resistance to permethrin (92 % mortality) (personal observation), while the resistant strain showed high level resistance to this insecticide (42% mortality) (Munhenga and Koekemoer, 2011).

Microarray experiments indicated that 29 genes were over-transcribed according to the criteria outlined in section 3.2.4 (Table 3.2). Most of these were CYP genes (55%), followed by redox genes (21%), and then GSTs (14%) (Figure 3.1). The "other" genes included cytochrome c, as well as a ribosomal gene and a receptor protein. The five genes with the highest transcript abundance were *CYP6AG2*, *CYP6Z1*, *TPX2*, *CYP6Z2* and *CYP6P1*, in order of statistical significance (i.e. adj. *p*-value) (Table 3.2).

**Table 3.2** List of over-transcribed probes in the resistant phenotype. Probes are listed in order of significance (adj. *p*-value), and then by fold change (FC) value. The accession number refers to Genbank, unless otherwise specified in the table. E = exponent.

| Gene | Function | FC | Adj. *p*-value | Accession number | Location |
|---|---|---|---|---|---|
| *CYP6AG2** | Cytochrome P450 | 4.1 | 2.58E-7 | AY745224 | 2R |
| *CYP6Z1 (oligo)* | Cytochrome P450 | 4.7 | 3.05E-7 | AF487535 | 3R |
| *TPX2* | Thioredoxin peroxidase | 2.3 | 4.56E-7 | TIGR: TC48596 | 3L |
| *CYP6Z2* | Cytochrome P450 | 3.6 | 1.14E-6 | XM_317252 | 3R |
| *CYP6P1** | Cytochrome P450 | 2.2 | 1.14E-6 | AY028785 | 2R |
| *GSTU1** | Glutathione S-transferase | 2.2 | 3.03E-6 | XM_309135 | X |
| *SOD2* | Superoxide dismutase | 3.5 | 3.81E-6 | AY524130 | 2L |
| *CYP12F2** | Cytochrome P450 | 2.9 | 3.81E-6 | AY176050 | 3R |
| *CYP6Y2* | Cytochrome P450 | 2.2 | 3.81E-6 | AY193728 | 3R |
| *GPR npy 3* | G protein coupled receptor | 3.2 | 4.83E-6 | ENSANG: G00000009317 | 2R |
| *CYP9J5** | Cytochrome P450 | 3.2 | 9.14E-6 | AY748830 | 3L |
| *GPX1* | Glutathione peroxidase | 2.4 | 9.14E-6 | AY842257 | 2R |
| *CYP6P3** | Cytochrome P450 | 2.6 | 1.68E-5 | AF487534 | 2R |
| *CYP6Z1 (cDNA)* | Cytochrome P450 | 3.0 | 1.96E-5 | AF487535 | 3R |
| *CYP6M3** | Cytochrome P450 | 4.5 | 1.99E-5 | AY193730 | 3R |
| *SOD1** | Superoxide dismutase | 2.7 | 2.35E-5 | AY505417 | 3L |
| *CYP6Z3* | Cytochrome P450 | 3.0 | 2.95E-5 | AY193727 | 3R |
| *CYP6AK1** | Cytochrome P450 | 3.6 | 5.42E-5 | AY745227 | 3L |
| *CYP9M1* | Cytochrome P450 | 2.6 | 1.03E-4 | AY748836 | 3R |
| *CYP12F4** | Cytochrome P450 | 2.3 | 1.68E-4 | AY176048 | 3R |
| *CYP4H24** | Cytochrome P450 | 2.6 | 2.05E-4 | AY062206 | X |
| *CYP6M2** | Cytochrome P450 | 4.6 | 3.38E-4 | AY193729 | 3R |
| *GSTD1-3* | Glutathione S-transferase | 2.0 | 6.83E-4 | AF071163 | 2R |
| *CYP6M1* | Cytochrome P450 | 2.2 | 1.16E-3 | AY062208 | 3R |
| *GSTS1-1** | Glutathione S-transferase | 2.5 | 1.64E-3 | L07880 | 3L |
| *RPS26* | Ribosomal protein | 3.1 | 2.16E-3 | EMBL: 4A3A-AAM-G-11-R | 3L |
| *GPX3* | Glutathione peroxidase | 2.1 | 2.16E-3 | AY745228 | X |
| *TPX4** | Thioredoxin-dependent peroxidase | 2.8 | 3.60E-3 | AY745235 | 3L |
| *GSTS1-2** | Glutathione S-transferase | 2.3 | 4.15E-3 | AF513639 | 3L |
| *Cytochrome_C* | Cytochrome c | 2.0 | 2.17E-2 | TIGR: TC48590 | 3R |

* These genes are shared with the pyrethroid and DDT resistant strain, MBN-DDT. Metabolic detoxification in MBN-DDT confers resistance to deltamethrin.

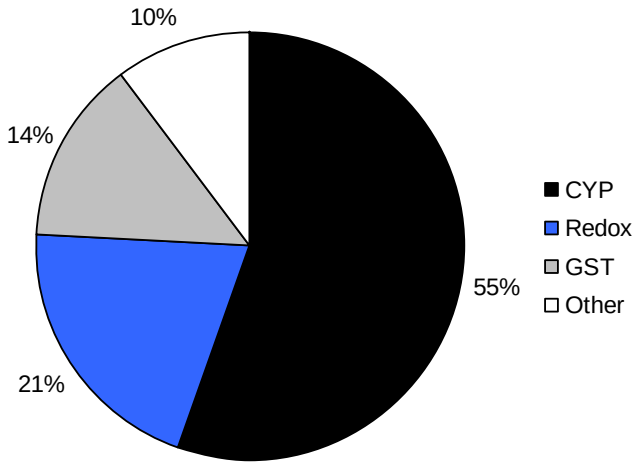



**Figure 3.1** Proportion of different gene families that are associated with permethrin resistance in KWAG-perm, according to microarray analyses.

Two genes were selected for qPCR validation of microarray data – *CYP6AG2*, because it was the most statistically significant gene, and *TPX2*, because the *TPX* genes have been found to be important in other instances of insecticide resistance (Müller et al., 2007b; Nardini et al., 2012). Furthermore, some of the other important genes (e.g. *CYP6Z1*, *CYP6Z2*, etc.) have already been analysed in an earlier study (Munhenga and Koekemoer, 2011). The genes, *CYP6AG2* and *TPX2*, produced fold change values of 2.9 and 4.5 respectively (Figure 3.2). An FC value of $\geq 2$ was considered to be significant.

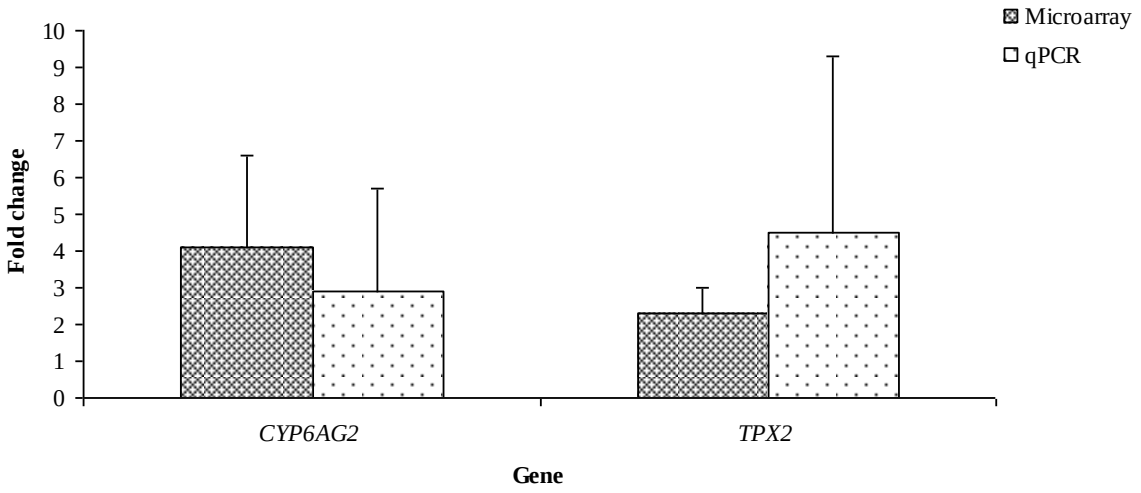



**Figure 3.2** A comparison between mean fold change (+SD) values recorded using microarrays and qPCR.

## 3.4 Discussion

In this study, detoxification enzyme gene expression in a permethrin resistant strain of *An. arabiensis* was investigated using the *An. gambiae* detox chip developed by David et al., (2005). Pyrethroids are widely used for vector control as they have low mammalian toxicity, are highly effective and are fast-acting (WHO, 1989; Coosemans and Carnevalle, 1995; Liu et al., 2006). Resistance to pyrethroids is therefore a serious problem and determining which genes are associated with protection against them is extremely important. Laboratory strains of insects are a valuable tool for gene-expression studies as they allow us to isolate a particular trait, and eliminate confounding parameters (that may or may not be known to the researcher). The laboratory strain used in this study was originally colonised from a wild population (KZN

province, South Africa) that showed resistance to permethrin. A sub-population was placed under continuous selective pressure (using permethrin) and was used for the present study.

Microarray analysis produced a list of 29 over-transcribed genes according to the criteria used (adj. *p*-value ≤ 0.05; FC ≥ 2). Most of the genes that were over-transcribed belong to the cytochrome P450 superfamily. This class of genes is frequently associated with pyrethroid resistance (Hemingway and Ranson, 2000; Brooke and Koekemoer, 2010). The top four P450 genes included *CYP6AG2*, *CYP6Z1*, *CYP6Z2* and *CYP6P1*. Most of the over-transcribed CYP genes belong to the CYP6 family, the members of which are well-known for their role in insecticide resistance (Scott, 1999; Nikou et al., 2003).

According to Mouatcho et al. (2009), permethrin resistance in *An. arabiensis* was correlated with an increase in total P450 activity/levels. These conclusions were based on synergist experiments and biochemical analysis (quantification of enzyme levels). The findings of the present microarray based survey support these conclusions. Recently, Munhenga and Koekemoer (2011) used qPCR to evaluate transcription of six genes that have been implicated in permethrin resistance in *An. arabiensis*. These included *CYP6Z1*, *CYP6Z2*, *CYP6Z3*, *CYP6M2*, *CYP6P3* and *CYP4G16*. Of these, five appeared on the list of over-transcribed genes in Table 3.2, but only three - *CYP6Z1* (4.7-fold), *CYP6Z2* (1.7-fold) and *CYP6M2* (2.2-fold) - were significantly over-transcribed in the qPCR-based study (Munhenga and Koekemoer, 2011).

*CYP6Z1* is constitutively over-expressed in pyrethroid resistant *An. gambiae* (Nikou et al., 2003) and *An. arabiensis* (Munhenga and Koekemoer, 2011). These data correlate with the findings of the present study in which *CYP6Z1* showed a 4.7 fold increase in expression relative to the susceptible strain (this FC value is comparable to that obtained in the cited studies). *CYP6Z2* has also been implicated in pyrethroid resistance in both *An. gambiae* (Müller et al., 2007a) and *An. arabiensis* (Munhenga and Koekemoer, 2011). Similarly, *CYP6Z2* was significantly over-transcribed in the present study (3.6 fold). However, the FC value obtained here is higher than that obtained by Munhenga and Koekemoer (2011) where a 1.7 fold increase in transcription was reported.

Another P450 that is encoded on the 3R chromosome arm is *CYP6M2*. This enzyme is over-transcribed in a number of pyrethroid resistant populations (Müller et al., 2007a, Djouaka et al., 2008; Munhenga and Koekemoer, 2011; Nardini et al., 2012), including the laboratory strain used here, and recently it was shown that *CYP6M2* is able to detoxify both permethrin and deltamethrin (Stevenson et al., 2011). In addition, this enzyme is able to metabolise DDT (Mitchell et al., 2012).

The most significantly over-transcribed gene, *CYP6AG2*, is not well characterised and this should be considered in future work. This gene is located on chromosome arm 2R, a region that is not typically linked to pyrethroid resistance. *CYP6AG1* was over-transcribed in a wild population of *An. arabiensis* from Cameroon that showed low-level resistance to deltamethrin (Müller et al., 2007b).

Pyrethroids are also known to induce oxidative stress by inducing lipid peroxidation, protein oxidation, and depletion of reduced glutathione (Vontas et al., 2001). This effectively increases the toxicity of the insecticide. A system of enzymes, that includes the SODs, catalases, peroxidases, cytochrome c and GSTs, is present which provides defense against these reactive oxygen species (ROS) (Bauer et al., 2002; James and Xu, 2012). If ROS are not metabolised, they damage important compounds such as lipids, proteins, nucleic acids and carbohydrates, and ultimately cause cell death (James and Xu, 2012). A number of redox enzymes showed elevated levels of transcription which may be associated with aiding resistance to pyrethroids. These included thioredoxin peroxidases (*TPX2* and *TPX4*), glutathione peroxidases (*GPX1* and *GPX3*), superoxide dismutases (*SOD1* and *SOD2*) and cytochrome c. Superoxide radicals are converted to hydrogen peroxide and oxygen by SODs, and hydrogen peroxide is converted to water and oxygen by catalases; or to water, by peroxidases (Fridovich, 1978). It is interesting to note that when mosquitoes are infected with *Plasmodium*, *SOD1* and *SOD2* are down-regulated at various stages of the parasite life-cycle (Félix et al., 2010). This is important given the fact that SODs are important in pyrethroid resistance.

Four GSTs (*GSTU1*, *GSTD1-3*, *GSTS1-1*, *GSTS1-2*) were over-transcribed in the resistant strain. Over-transcription of GSTs has been observed before in pyrethroid resistant *An. arabiensis* (Müller et al., 2007b; Nardini et al., 2012), *An. gambiae* (David et al., 2005; Müller et al., 2007a; Djouaka et al., 2008) and in other insects (Hemingway et al., 1993; Lagadic et al., 1993; Kostaropoulos et al., 2001). In conjunction with glutathione, GSTs function as antioxidants by limiting peroxidation and by limiting (termination) “free-radical

cascades" (Vontas et al., 2001, and references therein). In addition, the GSTs are able to bind to pyrethroids and provide protection by sequestration (Kostaropoulos et al., 2001).

In Nardini et al. (2012) (Chapter 2 of this thesis), the authors listed a set of genes that were over-transcribed in relation to deltamethrin resistance. All the P450s found to be over-transcribed in that study, also appear in Table 3.2, and there is some overlap in the GSTs that are over-transcribed. It is possible that the genes that are unique in each instance are more important for resistance to either a type I (e.g. *GSTD2, GSTD12*) or type II pyrethroid (*CYP6Z1, CYP6Z2*), while those that are common are likely to play a role in resistance to both kinds of pyrethroids.

Identifying multiple genes associated with metabolic pyrethroid resistance in a South African *An. arabiensis* population is only the beginning of our understanding of this complex resistance mechanism. Objectives for future work should include an assessment of whether the transcript abundance observed here is reflected in field populations with pyrethroid resistance, and if so, the role and importance of individual genes in pyrethroid resistance be determined.

# Chapter 4

## Effect of *Beauveria bassiana* infection on detoxification enzyme transcription in *Anopheles arabiensis*: a preliminary study

## 4.1 Introduction

Malaria vector control is largely based on indoor residual spraying and the use of insecticide treated bednets (WHO, 2011). However, these efforts have been undermined by the rapid spread of insecticide resistance (WHO, 2011). This has stimulated research into alternative vector control options. A sustainable and non-chemical approach would be ideal and with this in mind, the entomopathogenic fungi have received a great deal of attention in the context of both mosquito larvae and adults (Clark et al., 1968; Miranpuri and Khachatourians, 1990; Scholte et al., 2003; Achonduh and Tondje, 2008; Mohanty and Prakash, 2008; Farenhorst et al., 2009; Mnyone et al., 2009; Kikankie et al., 2010; Knols et al., 2010; Blanford et al., 2011; Mouatcho et al., 2011; Bilal et al., 2012).

Unlike bacteria and viruses, fungal entomopathogens do not need to be ingested in order to cause infection (Fang et al., 2012). This feature is advantageous in the case of blood-sucking insects like mosquitoes. The infection cycle has been outlined in detail in a number of publications (Charnley, 1989; Hegedus and Khachatourians, 1995; Clarkson and Charnley, 1996). During the infection process, the entomopathogenic fungi produce toxins that assist in attacking the insect host. These toxins are ionophoric, cyclic peptides that cause sluggishness and paralysis in the host. In addition, they are phytotoxic and antimicrobial (Liu and Tzeng, 2011). The fact that fungi are sometimes able to kill the host after only limited growth indicates that the toxins play an important role in the infection process (Roberts, 1980; Samuels et al., 1988).

One of the challenges associated with the use of fungal pathogens is the relatively slow speed of kill. However, a recent study reported median survival times of less than 4 days and 100% mortality within 6 days following exposure to *B. bassiana* (Blanford et al., 2011). Another study with more conservative results reported 100% mortality between 8 and 20 days (Farenhorst et al., 2009). In the context of malaria vector control, the fact that the parasite requires 10-14 days to develop within the mosquito (Charlwood et al., 1997) means that it is possible to achieve 100% mortality within this time frame using fungal pathogens. These promising results, along with the fact that infection causes a number of deleterious pre-lethal effects (Blanford et al., 2011; George et al., 2011), suggest that fungal pathogens have the potential to be successful biopesticides, particularly in integrated vector control programs. Furthermore, it has also been shown that fungal infection is detrimental to parasite development, significantly reducing transmission risk (Blanford et al., 2005).

A number of important interactions between entomopathogenic fungi and their hosts have been reported, especially in the context of disease vectors like mosquitoes. For example, dengue virus replication in the midgut of *Aedes aegypti* is hampered following infection of the mosquito with *B. bassiana* which activates certain immune pathways thereby disrupting viral replication (Dong et al., 2012).

Metabolic detoxification is one of the ways in which insects protect themselves from insecticides and it has been hypothesised that enhanced detoxification can interact with certain fungal virulence factors, such as toxins, and reduce their effect in the infection process (Serebrov et al., 2006; Farenhorst et al., 2009). This is an important consideration because if detoxification of these metabolites does occur, then fungal pathogens may not be as effective

as vector control agents. *Beauveria bassiana* produces a range of secondary metabolites such as bassianolide, beauvericin, beauverolides, oosporein, bassianin, tenellin and oxalic acid (Roberts, 1980; Hegedus and Khachatourians, 1995; Rohlfs and Churchill, 2011). Considerably more is known about the secondary metabolites of *Metarhizium anisopliae* and interestingly, it has been shown that some insects are able to detoxify some of these metabolites (Jegorov et al., 1992; Pedras et al., 2002).

Most studies on the subject have found that the presence of insecticide resistance in *An. gambiae*, *An. arabiensis* and *An. funestus* does not impact on susceptibility to fungal infection (Farenhorst et al., 2009; Blanford et al., 2011). However, a survival assay in *An. gambiae* reported reduced susceptibility to fungal infection in insecticide-susceptible mosquitoes when compared with their pyrethroid resistant counterparts (Howard et al., 2010). Presumably, this effect was related to the fact that insecticide resistance sometimes results in reduced fitness (Berticat et al., 2008). Another study has suggested that insecticide resistance mechanisms and the response to fungal infection may be linked. In this instance, *M. anisopliae* infection in the greater wax moth, *Galleria mellonella*, produced a total increase in GST and esterase activity and increased the resistance of the caterpillars to the organophosphate, malathion (Serebrov et al., 2006). In addition, biochemical assays using enzyme inhibitors produced an increase in insect death from fungal infection when compared with caterpillars that were treated only with fungal spores.

With the findings of Serebrov et al. (2006) in mind, and the fact that transcription levels of detoxification enzymes have not been directly measured in previous studies that deal with fungal infection in mosquitoes, the transcription levels of certain detoxification enzymes,

known to play an important role in pyrethroid resistance, were investigated following fungal exposure. In Chapter 2, microarrays were used to evaluate the detoxification enzyme profile of *An. arabiensis* with multiple resistance (Nardini et al, 2012). Data from this study was used to investigate the impact of fungal infection on transcription of selected genes in DDT and pyrethroid resistant *An. arabiensis*.

## 4.2 Materials and methods

### 4.2.1 Mosquito strains

The *An. arabiensis* strains used for the study, SENN-DDT and MBN-DDT, were both resistant to DDT and pyrethroids (see Chapter 2, section 2.3.1). SENN-DDT and MBN-DDT are fixed for the *kdr* mutation, L1014F, which is most likely to be the main mechanism for DDT and permethrin resistance, while the deltamethrin resistance was mainly associated with increased P450 activity (Chapter 2). The mosquitoes were reared as described in Chapter 2 (section 2.2.1).

### 4.2.2 Fungus formulation and mosquito exposures

Spore application was based on the method of Blanford et al. (2011). Briefly, BotaniGard® ES (BioWorks Inc., USA) a commercial liquid product based on conidia of *B. bassiana* strain GHA was further diluted in mineral oil (80% Isopar M: 20% Ondina 22). The formulation was applied to clay tiles (standard clay poured in to 15cm Petri dishes, and allowed to dry) using a

spray pump clamped horizontally over the tile at a fixed distance above the tile. Five pumps (0.7ml) produced an application rate of $5.5 \times 10^{11}$ spores/$m^2$ and the tiles were subsequently left to dry for 24 hours. The exposure protocol is based on the WHO cone bioassays (WHO, 2006). Clear plastic cones, with openings at the top, were placed in the centre of each tile and plugged with cotton wool (Figure 4.1). Approximately 25, three day old female *An. arabiensis* were placed in each cone for 30 minutes. Four replicates were prepared and the experiment was repeated four times. Following exposure, the mosquitoes were transferred to paper cups covered with netting and were maintained on a 10% sugar water solution. Mortality was recorded daily for 14 days and dead mosquitoes were removed each day. Controls included exposures to clay tiles only (i.e. not sprayed at all) and exposures to tiles sprayed with oil only. As above, four replicates were prepared each time the experiment was carried out (four times), and approximately 25 mosquitoes were used for each replicate. MBN-DDT and SENN-DDT exposures were run in parallel each time a repeat was prepared. Bioassay data were analysed using Kaplan-Meier Survival Curves and differences in median lethal times (MLTs) was determined using a Log Rank test in SPSS® Statistics 17.0 (2008). In each repeat, one replicate (i.e. one cup) was sacrificed for RNA extractions, so the analyses were done on the three remaining replicates for each treatment group.

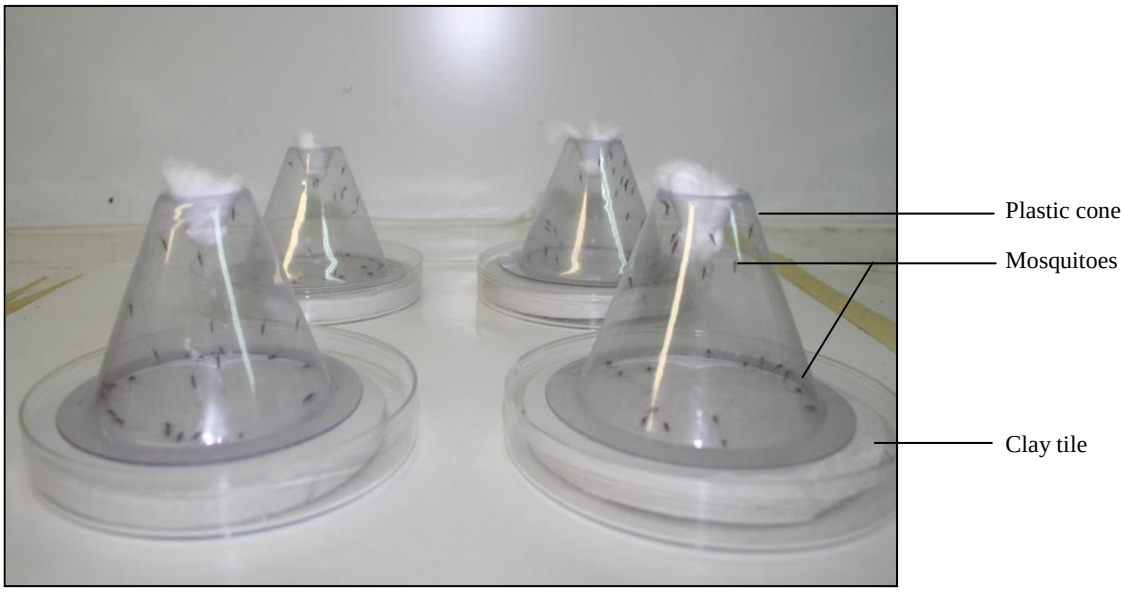



**Figure 4.1** Set-up of cone bioassays. Clear plastic cones were placed on the centre of clay tiles that were sprayed with spores of *B. bassiana*. Mosquitoes were placed inside the cones and exposed to the spores for 30 minutes before being transferred to paper cups for monitoring.

### 4.2.3 RNA extractions

Four days post infection (dpi), one replicate was removed from the experiment and RNA was extracted from a sub-sample of 12 mosquitoes, representing one biological repeat (the remainder of the mosquitoes in this replicate were discarded). In addition, RNA was extracted from mosquitoes exposed to the clay plus oil control. RNA was extracted using TRI® Reagent solution (Sigma-Aldrich) according to the method described in section 2.2.9 (Chapter 2). Samples were quantified using a NanoDrop spectrophotometer, the quality of the RNA confirmed by gel electrophoresis (Chapter 2, section 2.2.6), and then reverse transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen) according to supplier instruction. cDNA was stored at -20°C until required for PCR.

### 4.2.4 Quantitative real-time PCR (qPCR)

For SENN-DDT, three genes were evaluated by qPCR - *CYP6M2*, *CYP9L1* and *CYP4G16* (Nardini et al., 2012); and for MBN-DDT, *CYP6M2*, *CYP9L1* and *TPX4*. The primer sequences used were the same as those in Chapter 2. A reference gene evaluation was done for each strain in order to select the most stable reference gene for relative quantification. This was done using NormFinder (2004, Molecular Diagnostic Laboratory, Aarhus University Hospital) and the following candidate genes were tested: ribosomal *sp 7 (rsp 7)*, ribosomal protein L19 (*RPL19*), the cytoskeletal protein *β-actin*, *GAPDH* and TATA binding protein. For SENN-DDT, gene expression was measured relative to *RPL19*, and for the MBN-DDT, gene expression was measured against *rsp 7* (it is important to note that reference gene selection is required each time a study is conducted under new conditions e.g. following fungal exposure, and so reference genes were not the same as those used in Chapter 2). PCR was carried out using the Bio-Rad CFX96™ Real-Time PCR Detection System. Each reaction was set up as described in Chapter 2 (section 2.2.9). Primer sequences, annealing temperatures and concentrations are described in Table 4.1. Standard curves were prepared by two-fold dilutions of cDNA derived from mosquitoes that were exposed to *B. bassiana*. Three biological repeats were prepared, and for each biological repeat, three technical repeats were included. Data were analysed using the Pfaffl (2001) method. Initially, PCR product for each gene of interest was sent to Macrogen Europe for sequencing in order to confirm that the correct product was amplified. The small amplicons (<110 bp), *CYP4G16* and *RPL19*, were cloned before sequencing, as described in Chapter 2 (section 2.2.10).

**Table 4.1** SENN-DDT and MBN-DDT primer information for qPCR. All products were cloned to ensure that the desired target was amplified (bp = base pairs).

| Gene | Primer sequence | Primer concentration | Annealing temperature | | Amplicon length |
|---|---|---|---|---|---|
| | | | SENN | MBN | |
| *CYP9L1* | Fwd 5'-AGA TAA TGT ATT CTT TCG CTA TGG-3' | 3.5µM | 56.3°C | 58.3°C | 188 bp |
| | Rev 5'-GCT CTT CTC GCT CTT GAA C-3' | | | | |
| *CYP6M2* | Fwd 5'-CAT GAC ACA AAC CGA CAA GG-3' | 3.5µM | 60.0°C | 60°C | 235 bp |
| | Rev 5'-GGT GAG GAG AGT CGA CGA AG-3' | | | | |
| *CYP4G16* | Fwd 5'-CAG ACC GTC CAG CCA CAT TC-3' | 3.0µM | 59.4°C | - | 108 bp |
| | Rev 5'-GCG AAC GAG CAA TTA TAG GTA CTG-3' | | | | |
| *TPX4* | Fwd 5'-CAG CTG ACA GAC CGA TTA AG-3' | 3.5µM | - | 58.3°C | 116 bp |
| | Rev 5'-CCG TTC GGG AAC AGT TTG TCT-3' | | | | |
| *RPL19* | Fwd 5'-CCA ACT CGC GAC AAA ACA TTC-3' | [a] | | [b] | 61 bp |
| | Rev 5'-ACC GGC TTC TTG ATG ATC AGA-3' | | | | |
| *rsp 7* | Fwd 5'-TTA CTG CTG TGT ACG ATG CC-3' | [a] | [b] | | 135 bp |
| | Rev 5'-GAT GGT GGT CTG CTG GTT-3' | | | | |

[a] Concentration used was dependent on that of target genes

[b] Temperature used was dependent on annealing temperature of target genes

## 4.3 Results

When adult *An. arabiensis* females were exposed to *B. bassiana* spores for 30 minutes, first mortality from mycosis was observed two days post infection (dpi) and 100% mortality was obtained by 13 dpi for both strains (Figure 4.2). The median lethal time (MLT) for both SENN-DDT and MBN-DDT was 6 days (Table 4.2). The log rank statistic showed that the effect of *B. bassiana* infection on survival was significant in the exposed cohorts ($p < 0.001$).

**Table 4.2** Survival estimates SENN-DDT and MBN-DDT strains exposed to *B. bassiana*. Estimates of time to 100% mortality are the mean of the four replicates in each treatment group. N/A indicates that the survival estimate (50% or 100% mortality) was not achieved across the study period.

| **Strain** | **Median lethal time (± 95% CI) days** | **Time to 100% mortality (days)** | **Log rank statistic** | **Significance** |
|---|---|---|---|---|
| SENN-DDT | 6.0 (5.7 – 6.3) | 13 | - | - |
| Clay control | N/A | - | 643.9 | $p < 0.001$ |
| Clay + oil control | N/A | - | 719.3 | $p < 0.001$ |
| MBN-DDT | 6.0 (5.8 – 6.2) | 13 | - | - |
| Clay control | N/A | - | 532.4 | $p < 0.001$ |
| Clay + oil control | N/A | - | 691.9 | $p < 0.001$ |

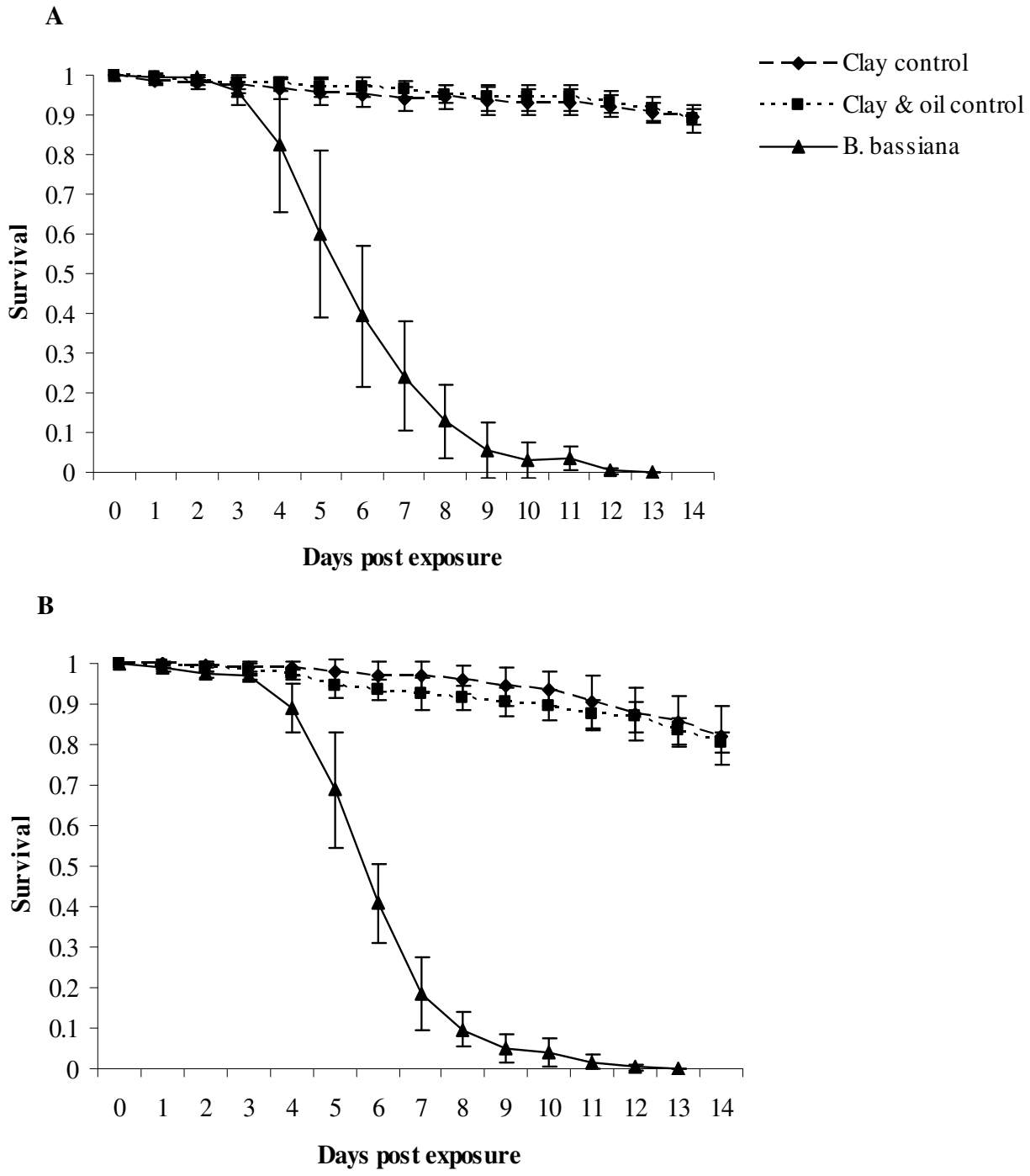



**Figure 4.2** Mean (±SD) cumulative proportional survival of (A) SENN-DDT and (B) MBN-DDT *An. arabiensis* mosquitoes infected with *B. bassiana*. Data represents 4 repeats (3-4 replicates per repeat, each containing approximately 25 mosquitoes).

To investigate whether fungal exposure affected the transcription of the selected detoxification genes, qPCR was used and transcription was measured relative to that of a stable reference gene to obtain a fold change (FC) value. The FC values obtained here were compared with those obtained in microarray experiments performed in Chapter 2, and FC values of more than 1.5 were considered to be significant. In the case of SENN-DDT, no genes were over-transcribed above this value. For *CYP6M2*, *CYP9L1* and *CYP4G16*, FC values of 1.1, 0.8 and 0.9 respectively were obtained (Figure 4.3). The outcome was similar for MBN-DDT where FC values of 1.3, 0.8 and 0.6 were obtained for *CYP6M2*, *CYP9L1* and *TPX4* respectively (Figure 4.4).

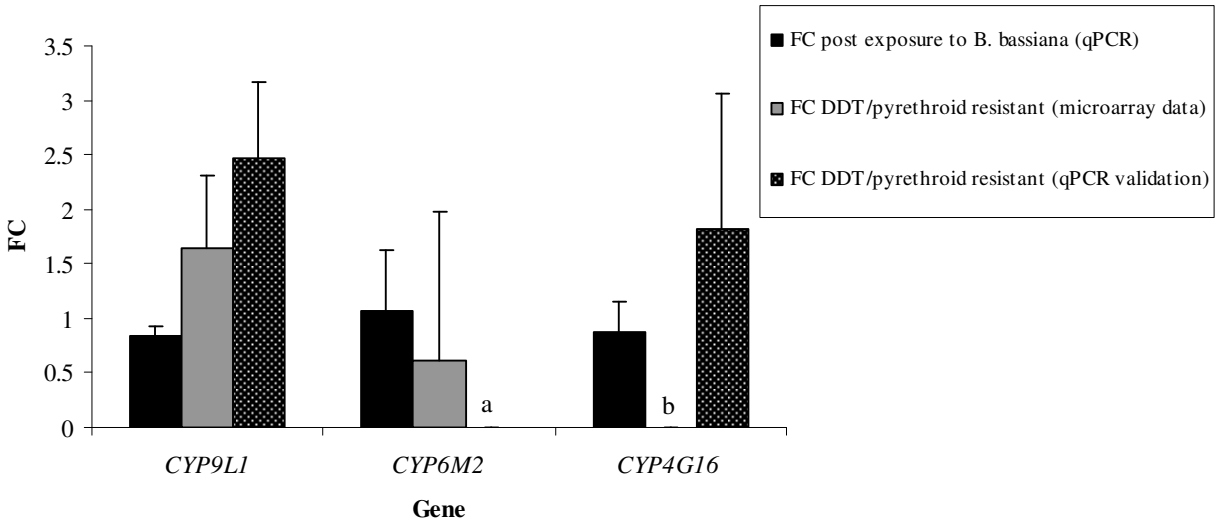



**Figure 4.3** Transcription levels of important genes involved in pyrethroid resistance, following exposure to *B. bassiana* in SENN-DDT. The FC cut-off for significance is ≥ 1.5. Black bars represent FC values, obtained by qPCR, of samples infected with *B. bassiana* (relative to uninfected samples); grey bars represent FC values of insecticide resistant samples, measured relative to susceptible samples, by microarray analysis (as per Chapter 2); and shaded bars represent FC values of insecticide resistant samples, measured relative to susceptible samples, by qPCR analysis (as per Chapter 2). a = no qPCR validation data as this gene was not over-transcribed in SENN-DDT, b = no microarray data due to saturation (saturated spots not included in analysis).

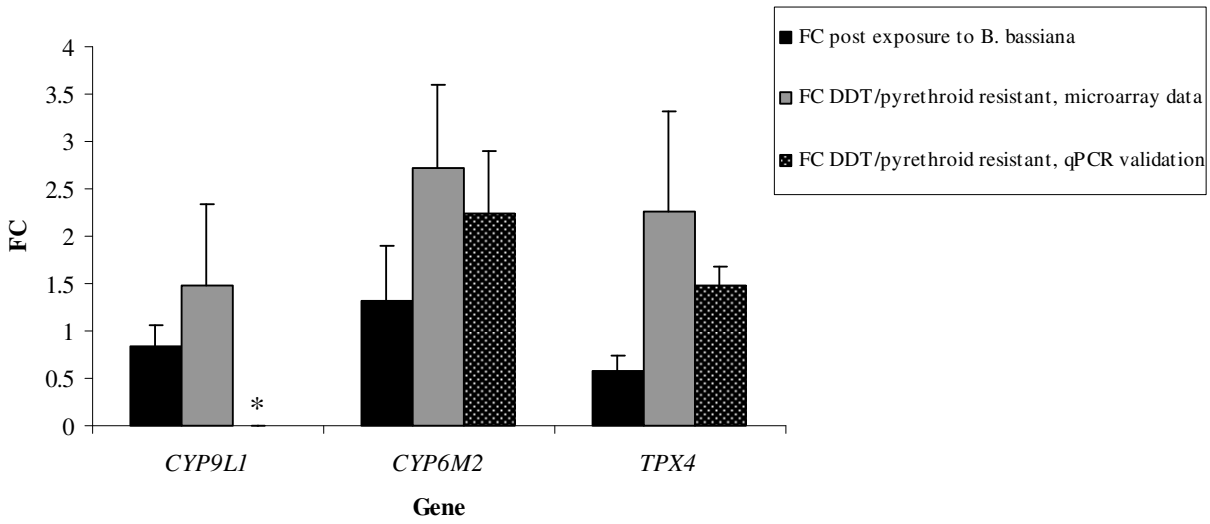



**Figure 4.4** Transcription levels of important genes involved in pyrethroid resistance, following exposure to *B. bassiana* in MBN-DDT. The FC cut-off for significance is ≥ 1.5. Black bars represent FC values, obtained by qPCR, of samples infected with *B. bassiana* (relative to uninfected samples); grey bars represent FC values of insecticide resistant samples, measured relative to susceptible samples, by microarray analysis (as per Chapter 2); and shaded bars represent FC values of insecticide resistant samples, measured relative to susceptible samples, by qPCR analysis (as per Chapter 2). * = no validation data as this gene was not over-transcribed in MBN-DDT.

## 4.4 Discussion

The strain of *B. bassiana* used here proved to be highly infective and virulent to insecticide resistant *An. arabiensis*. Median lethal times (MLTs) of six days for both SENN-DDT and MBN-DDT mosquito strains and 100% mortality within the 14 day monitoring period is in line with a number of other studies reporting the survival impact of *B. bassiana* on mosquito vectors of malaria (Achonduh and Tondje, 2008; Farenhorst et al., 2009; Mnyone et al., 2009; Kikankie et al., 2010; Blanford et al., 2011).

One of the key benefits of using insecticides for malaria vector control is that they kill the host quickly following contact with sprayed or protected surfaces, effectively blocking transmission of the parasite. In order for malaria vectors to transmit *P. falciparum*, the parasite requires at least 10 days inside the mosquito to complete its developmental cycle before it can be transmitted to a human host as sporozoites at a temperature of 25°C (WHO, 1975) and shorter, when the temperature is warmer (Beier, 1998). Fungal pathogens do not cause immediate knockdown, but are able to kill the host within the required parasite development period, suggesting that they could be used effectively to prevent transmission (Blanford et al., 2011). In addition, fungal pathogenesis increases the time required for *Plasmodium* maturation in the mosquito (Blanford et al., 2005). These benefits are further enhanced by the fact that numerous pre-lethal effects have been reported including elevated metabolic rate, reduced flight capacity and reduced tendency to feed (Blanford et al., 2011; George et al., 2011).

Insect-pathogen interactions have been explored in great detail in the context of pathogen elimination from the host, but less attention has been paid to the process of detoxifying secondary metabolites of the pathogen or products that come from tissue damage during the infection process (Serebrov et al., 2006). In the case of fungal pathogenesis, these may be equally important as the entomopathogenic fungi produce an array of toxins during the infection process. To investigate whether fungal infection had an effect on transcription of genes associated with pyrethroid resistance, and hence the potential for these genes to affect fungal pathogenesis in resistant mosquitoes, transcription in a subset of important genes was assessed by qPCR. RNA was extracted from cohorts of 12 mosquitoes four days post infection (dpi). The choice to extract at this point was based on preliminary data where it was observed

that significant levels of mortality occurred from 5 dpi onward. This raised concern that any over-transcription, if present, would not be detected by qPCR as the mosquitoes were either dead or very inactive at this point. That said, it is important to note that toxin production can occur at different stages of the infection cycle. According to Charnley (1989), disruption of cells in the area of advancing hyphae has been reported, suggesting the presence of toxins. However, toxin production usually occurs during the yeast phase of the fungus in the haemocoel (Roberts, 1980). Pekrul and Grula (1979) conducted a detailed investigation of infection of the corn earworm, *Heliothis zea*, by a highly pathogenic strain of *B. bassiana*. These authors found that penetration of the cuticle occurred within 16 hours post infection (hpi), and by 48 hpi, extensive fungal growth was observed in the haemocoel. Infection of tissue was reported from 60-72 hpi. In the present study, mortality in the exposed mosquitoes began to deviate noticeably from the controls at 4 dpi suggesting that the fungus, and toxin production, is active at this time point, and for this reason, the RNA sampling time was selected.

Of the four genes assessed (three per strain), none showed an increase in transcription relative to the uninfected samples. The susceptible strains were not included in the study. However, for future work, this should be considered. It is possible that infection of the susceptible strains with *B.bassiana* may have produced significant changes in transcription that were not seen in resistant mosquitoes as genes that are already over-transcribed may not be further induced (and no changes in transcription will be observed).

The three cytochrome oxidases that were assessed have been correlated with metabolism of insecticides in *An. arabiensis* (Müller et al., 2007b; Nardini et al., 2012 [Chapter 2]), while the

fourth gene that was investigated was the thioredoxin peroxidase (*TPX4*) which was associated with the resistant phenotype in MBN-DDT (Chapter 2), as well as in other populations of *An. arabiensis* (Müller et al., 2007b). This is a redox enzyme typically involved in reducing oxidative stress – in this case, potentially to "clean-up" post P450 metabolism (which sometimes results in the production of reactive oxygen species), or alternatively, as protection during pathogenesis. Insect immunity is based on both humoral and cellular defense systems. The latter is of interest because it involves, amongst other things, the production of a range of cytotoxic reactive intermediates of oxygen which should aid in elimination of the pathogen (Nappi and Ottaviani, 2000). Given the likely presence of damaging oxygen molecules following exposure to a fungal pathogen, it is interesting that *TPX4* showed no increase in transcription.

It is likely that *B. bassiana* does produce toxins *in vivo* given that GHA is a virulent strain, able to produce oosporein, beauvericin and bassionolide (S. Jaronski, personal communication). It is possible that the lethal dose used in the present study resulted in very high levels of toxin production that "overwhelmed" the mosquitoes. Furthermore, only four detoxification enzymes were examined in this study. Given the size of these enzyme classes, other metabolic enzymes may well be involved in the host's response to infection. It would be valuable to apply microarrays to evaluate over-transcription of a broader range of genes following fungal infection (the microarrays used in Chapters 2 and 3 were no longer available when this study was conducted). It would also be valuable to include additional time points at which to evaluate of gene transcription.

The outcome of this study supports that of others but the conclusions of these were based on bioassays that involved exposure of insecticide resistant and susceptible mosquitoes to a fungal preparation. These authors found no significant benefit of resistance for protection against fungal infection (Farenhorst et al., 2009; Kikankie et al., 2010; Blanford et al., 2011). Furthermore, some studies have reported that exposure of a resistant mosquito to both insecticide and fungus increases the susceptibility of a normally resistant mosquito to the insecticide (Farenhorst et al., 2010).

It is, however, interesting to note that in some other insect species, evidence of detoxification of fungal metabolites has been reported (Serebrov et al., 2006). When *G. mellonella* larvae were exposed to *M. anisopliae*, GST, esterase and phosphatase activity increased. The authors suggest that this occurred as a non-specific response to physical damage by the fungal pathogen, or in response to the presence of fungal metabolites. In addition, the same authors found that exposure to pyrethroids following infection with *M. anisopliae* led to reduced susceptibility of the host to insecticide. In this study (Serebrov et al., 2006), it wasn't clear whether a lethal or sub-lethal dose was used. Furthermore, *G. mellonella* larvae are able to survive injection with the *M. anisopliae* toxin, destruxin, whereby after a period of paralysis, they recover fully (Jegorov et al., 1992). This is likely due to detoxification processes, but their recovery from toxin exposure is probably also dependent on the dosage of toxin injected (i.e. it is easier to detoxify and recover from low doses of toxin). It is possible that the lethal dose used in the present study resulted in very high levels of toxin production that "over-whelmed" the mosquitoes.

The entomopathogenic fungi have enormous potential for use as biocontrol agents. They act as contact insecticides which is particularly beneficial in the case of insects like mosquitoes. They are relatively easy to produce, even on a large-scale (St. Leger et al., 2011) and the fact that some, like the isolate used in this study, are already in commercial production means that it could possibly be used in a vector control context but with a reduction in research and development requirements associated with a completely novel product (Blanford et al., 2011). Genetic engineering is being used to improve the pathogenesis of these fungi so that efficacy can be improved (Fang et al., 2012; Shang et al., 2012).

# Chapter 5

# Discussion and Conclusion

## 5.1 General Discussion

*Anopheles arabiensis* is one of the most important malaria vectors in Africa (Sinka et al., 2012), and an important vector of malaria in South Africa (Coetzee et al., 2000). While much is known about insecticide resistance in *An. gambiae*, less attention has been paid to *An. arabiensis*, and part of the goal of this research was to help bridge the information gap. Pyrethroid and DDT resistance in *An. arabiensis* are well documented, and is of great concern in areas of high malaria transmission. Sustainable and effective vector control is dependent on a clear and comprehensive understanding of the mechanisms involved in conferring resistance (WHO, 2012). Metabolic-based mechanisms are a common cause of resistance, and are based on three large enzyme families – the cytochrome monooxygenases/P450s, the glutathione S-transferases (GSTs), and the esterases. Target-site resistance mechanisms are also important. One of the most common is knockdown resistance (*kdr*) which is a mutation in the voltage gated sodium channel and which confers resistance to both DDT and pyrethroids.

The aims of this study were to evaluate, in detail, the basis of DDT and pyrethroid resistance in *An. arabiensis*, with particular emphasis on metabolic detoxification. This was done using laboratory strains so that the impact of complicating factors could be minimised (for example, exposure to agricultural insecticides is a factor when one is studying wild populations of insects). In addition, the impact of *Beauveria bassiana*, an important candidate for biological control of mosquitoes, on mosquito gene expression was evaluated.

**5.2 Which detoxification enzymes are associated with DDT and pyrethroid resistance in laboratory strains of *Anopheles arabiensis* of different geographic origin?**

The study of detoxification enzyme profiles of two insecticide resistant strains of *An. arabiensis* from two different regions in Africa using the *An. gambiae* detoxification microarray (David et al., 2005) produced some interesting results. Although both strains showed phenotypic similarities, the microarray data revealed that only one gene, *CYP9L1*, was over-transcribed in the Sudanese strain; while in the South African strain, 20 genes were over-transcribed. Most of these were P450s, followed by GSTs and a small number of redox genes. Amongst the over-transcribed genes were *CYP6M2*, *CYP6AK1*, *CYP6P3*, *TPX4* and *GSTS1-2* which have previously been implicated in pyrethroid resistance in *An. gambiae* (David et al., 2005; Müller et al., 2007a; Djouaka et al., 2008) and in one study of *An. arabiensis* from Cameroon (Müller et al., 2007b). A selection of other genes that were over-transcribed are less well-known and their role in resistance should be investigated further.

The synergist assays were used to clarify the role of the different enzyme classes in resistance to a particular insecticide. These assays suggested that the over-transcription of detoxification enzymes found in this study was related to deltamethrin resistance, while DDT and permethrin resistance were mainly associated with the presence of the *kdr* mutation. Permethrin and deltamethrin are type I and type II pyrethroids respectively, and differ in their chemical structure. The above is in keeping with the observed trend in *An. gambiae* that DDT resistance is very closely linked to *kdr* mutations, less so with permethrin, and the weakest link exists between deltamethrin resistance and *kdr* (Brooke, 2008; Brooke and Koekemoer, 2010).

Often, pyrethroids are discussed in general terms, and the differences, if any, between deltamethrin and permethrin resistance is vague or not clarified at all.

The fact that a number of GSTs were over-transcribed in MBN-DDT is likely to be related to the fact that pyrethroids directly induce oxidative stress, and P450 metabolism often produces reactive oxygen molecules, adding to the oxidative stress. GSTs are able to protect mosquitoes against this, although they are also involved directly through sequestration (Che-Mendoza et al., 2009).

These data emphasise the complexity associated with resistance phenotypes and suggest that specific insecticide resistance mechanisms cannot be extrapolated to different vector populations of the same species even if they have the same resistant phenotype. The difference in the number of genes that were found to be significant according to microarray analysis was striking given that the resistance phenotypes are similar and that metabolic detoxification is the main mechanism responsible for deltamethrin resistance. In studies based on laboratory reared insects, it is important to consider the impact of colonisation on phenotype (e.g. reproductive parameters) and genotype (e.g. changes in allele frequency, reduced heterozygosity) (Mason et al., 1987). It is possible that these factors may have contributed to the differences observed.

### 5.3 What detoxification enzymes are associated with permethrin resistance in a laboratory strain of *Anopheles arabiensis* from South Africa?

The data presented in Chapter 3 confirm that permethrin resistance can be based on enzymatic detoxification involving many enzyme systems. In the case of SENN-DDT and MBN-DDT (Chapter 2), permethrin resistance was induced by cross-resistance to DDT and was largely based on the presence of the L1014F mutation. The strain, KWAG-perm, however, showed no cross-resistance to DDT. This strain was derived from a permethrin resistant population and was maintained by weekly exposure to permethrin in the laboratory. More than half of the over-transcribed genes in each instance (MBN-DDT and KWAG-perm, both originally from the same locality in KZN, South Africa) were common to both MBN-DDT and KWAG-perm. This is interesting because in the case of the former, metabolic detoxification is responsible for deltamethrin resistance, while in the latter, metabolic detoxification is responsible for permethrin resistance. This suggests that a number of the over-transcribed genes can confer resistance to both types of pyrethroid and act in concert to produce a protective resistant phenotype. However, the genes that were unique to each colony may be unique for detoxification of a type I or type II pyrethroid. The functionality of these genes should be determined to see if this is the case. The possibility that unique genes confer resistance to different types of pyrethroid would be helpful to vector control efforts as one would be able to use alternative pyrethroids for control, where existing resistance to one type occurs. Generally, where permethrin resistance is observed, pyrethroids are no longer used for vector control and current resistance management protocol recommends alternating between different classes of insecticide (WHO, 2012). However, if the role of individual genes associated with resistance

to different pyrethroid types is clarified, it might be possible to modify this recommendation so that the lifespan of insecticides be extended.

### 5.4 Does *Beauveria bassiana* infection have an impact on detoxification enzyme transcription in *Anopheles arabiensis*?

The aim of this study was to determine whether genes associated with pyrethroid resistance in two laboratory-reared *An. arabiensis* strains are affected following exposure to the entomopathogenic fungus, *B. bassiana*. This entomopathogen has been studied for vector control as it provides a means of sustainable and environmentally friendly alternative to insecticides. Using data collected in microarray experiments (Chapter 2), a selection of important insecticide-resistance genes were studied in the context of *B. bassiana* infection. This is important to know because if *B. bassiana* does induce over-transcription of genes already associated with resistance, resistant mosquitoes will be less susceptible to fungal infection as they are already equipped with mechanisms that may hinder infection. The transcription of four genes, *CYP9L1*, *CYP6M2* and *CYP4G16* (cytochrome P450s) and *TPX4* (thioredoxin peroxidase) was investigated using real-time PCR. Overall, the fold change values for each gene were lower when compared with the uninfected resistant *An. arabiensis* and fungal infection did not further enhance the insecticide resistance phenotype. This is a positive outcome considering that pre-existing metabolic resistance is often found in wild mosquito populations. That said, it would be ideal to use the microarrays to compare a wider range of genes, and gene families. It would also be valuable to study the fungal toxins in greater detail. Information about the toxin production of *B. bassiana in vivo* is lacking and provides another avenue for research.

## 5.5 Conclusion

In this study, the resistance profiles of DDT and pyrethroid resistant *An. arabiensis* have been compared to that of susceptible strains. While DDT resistance was related to *kdr* in this instance (it can also be conferred by over-transcription of *GSTE2* [Ortelli et al., 2003]), pyrethroid resistance was more complicated as it was based on the presence of *kdr* and metabolic detoxification. Furthermore, the data suggest that there can be differences in the enzyme systems responsible for type I and type II pyrethroid resistance. Pyrethroid resistance was largely based on the over-transcription of P450s, but the GSTs, and a suite of antioxidant genes, were also important. Pyrethroids are the most commonly used class of insecticides, and in the case of mosquito vector control, are the only class of insecticide approved for use on bednets. The data presented here emphasise the fact that novel insecticide targets and/or control approaches are required for sustainable vector control. In this light, the fact that entomopathogenic fungi like *B. bassiana* are highly virulent to *An. arabiensis*, and given that in the important insecticide resistance genes tested here, they did not appear to enhance transcription, suggests that they are viable alternatives to insecticides.

# Appendix A[1]

[1] Published article based on Chapter 2

Parasites & Vectors

RESEARCH Open Access

# Detoxification enzymes associated with insecticide resistance in laboratory strains of *Anopheles arabiensis* of different geographic origin

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## Abstract

**Background:** The use of insecticides to control malaria vectors is essential to reduce the prevalence of malaria and as a result, the development of insecticide resistance in vector populations is of major concern. *Anopheles arabiensis* is one of the main African malaria vectors and insecticide resistance in this species has been reported in a number of countries. The aim of this study was to investigate the detoxification enzymes that are involved in *An. arabiensis* resistance to DDT and pyrethroids.

**Methods:** The detoxification enzyme profiles were compared between two DDT selected, insecticide resistant strains of *An. arabiensis,* one from South Africa and one from Sudan, using the *An. gambiae* detoxification chip, a boutique microarray based on the major classes of enzymes associated with metabolism and detoxification of insecticides. Synergist assays were performed in order to clarify the roles of over-transcribed detoxification genes in the observed *resistance* phenotypes. In addition, the presence of kdr mutations in the colonies under investigation was determined.

**Results:** The microarray data identifies several genes over-transcribed in the insecticide selected South African strain, while in the Sudanese population, only one gene, *CYP9L1*, was found to be over-transcribed. The outcome of the synergist experiments indicate that the over-transcription of detoxification enzymes is linked to deltamethrin resistance, while DDT and permethrin resistance are mainly associated with the presence of the L1014F *kdr* mutation.

**Conclusions:** These data emphasise the complexity associated with resistance phenotypes and suggest that specific insecticide resistance mechanisms cannot be extrapolated to different vector populations of the same species.

**Keywords:** *Anopheles arabiensis*, Insecticide resistance, Microarrays, Detoxification enzymes, *kdr*

## Background

In 2009, the World Health Organization (WHO) estimated 225 million cases of malaria worldwide [1]. Of these, 800 000 cases resulted in death, and most of these deaths occurred in Africa where infants, young children and pregnant women were, and still are, worst affected [1]. Insecticide use has been the most successful way of controlling malaria vectors, and as such, controlling the disease. As a result, the development of insecticide resistance in vector populations has had a major impact on malaria transmission and control.

*Anopheles arabiensis* is one of the major African malaria vectors and belongs to the *An. gambiae* complex. Resistance in this species has been reported in a number of countries and to a range of insecticides. Examples include dichlorodiphenyltrichloroethane (DDT), deltamethrin and permethrin resistance in Ethiopia [2,3]; partial resistance to permethrin in Tanzania [4]; DDT, permethrin, malathion and bendiocarb resistance in Sudan [5,6];

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DDT and permethrin resistance in South Africa [7,8]; and resistance to propoxur in Mozambique [9].

Insecticide resistance is either based on an increase in levels of detoxification enzymes [10], or is related to reduced target-site sensitivity [10,11]. Detoxification enzymes that are associated with insecticide resistance belong to large enzyme families, known as super-families. In *An. gambiae* there are multiple cytochrome P450s (n = 111) [12-16], esterases (n = 51) [14,15,17] and 31 glutathione S-transferase (GSTs) genes [14,15,17]. Numerous genes form part of these families and for this reason, it is difficult to determine the specific gene(s) associated with resistance to a particular insecticide, or class of insecticides. The development of high throughput technology such as microarrays provided a solution to this problem [18]. The *An. gambiae* detoxification microarray is a custom–made boutique array that includes GSTs, esterases and P450s as well as number of redox genes that are associated with P450 metabolism and which protect against free radical damage [19].

In addition to detoxification enzyme mediated protection against insecticides, a number of target-site resistance mechanisms are known. One of the best studied mechanisms in *An. gambiae* is *'kdr'* or knockdown resistance. This mechanism is characterized by a mutation in the voltage gated sodium channel that confers resistance to both DDT and pyrethroids [2,20-25]. However, the relationship between *kdr* and cross resistance between insecticide classes is not as clear cut as previously assumed [26]. In *An. gambiae* for example, the presence of *kdr* is most strongly correlated with DDT resistance, followed by permethrin resistance, while the weakest association is with the deltamethrin resistant phenotype [27].

In the original study in which the 'detox chip' was presented, the expression profile of detoxification genes associated with DDT resistance in a laboratory colony of *An. gambiae* was determined [19]. Genes that were over-transcribed included *GSTE2,* a gene that has previously been linked to DDT resistance [28,29], as well as *CYP6Z1, PX13A, PX13B* and *CYP12F1.* Since then, the detox chip has been used in several other studies. More recently, permethrin resistance in a wild *An. gambiae* population was monitored using the detox chip [30]. Three P450s showed high expression levels: *CYP6P3, CYP4H24* and *CYP4H19.* Although the detox chip was constructed using *An. gambiae* sequence information, it has been used with success in a number of cross-species hybridizations with *An. arabiensis* [31], *An. funestus* [32] and *An. stephensi* [33].

The aim of this study was to compare the transcription of detoxification enzymes of two laboratory strains of insecticide-resistant *An. arabiensis.* The colonies were originally derived from different geographic locations, one from Sudan and the other from South Africa. In Sudan, vector control includes the use of long lasting insecticide-treated bed nets (LLINs), temephos for larviciding, and bendiocarb is used for IRS [34]. South African vector control approaches include the use of IRS with DDT in traditional unplastered mud, grass or wooden houses and pyrethroids on walls with enamel painted surfaces [35,36].

## Methods

### Mosquito colonies

Mosquitoes were maintained under standard insectary conditions of 26 ± 2°C, a relative humidity of 70-80%, with a 12:12 light:dark cycle and 45 minute dusk/dawn period. The strains used for this study were as follows: *An. arabiensis,* colonized in the 1980's from the Sennar region of Sudan (SENN) and *An. arabiensis,* colonized in 2002 from the KwaZulu-Natal (KZN) province in South Africa (MBN). For each colony, both a susceptible or "unselected" strain (called the "base colony") was available, as well as a DDT-resistant strain. The resistant strains have been under continuous DDT selection from the time of colonization. To maintain resistance in the selected colonies, three day old adults were exposed to 4% DDT in every generation using World Health Organization (WHO) insecticide tubes and procedures [37]. Both DDT selected strains from Sudan and South Africa showed very low or no mortality (after 24 hr recovery period), following exposure to DDT for 1 hr and both were homozygous for the L1014F *kdr* mutation, as confirmed by PCR using AGD1 and AGD2 primers [23], and sequencing in both directions (data not shown). All strains are maintained in separate insectary rooms to minimise the chance of contamination between strains.

### World Health Organization insecticide susceptibility assays

The insecticide resistance status of the colonies were evaluated against a range of insecticides including DDT (4.0%), permethrin (0.75%), deltamethrin (0.05%), bendiocarb (0.1%), propoxur (0.1%) and fenitrothion (1.0%). The assays were done in order to confirm the resistance status of each strain. Assays were performed according to standard WHO procedures [37].

### Synergist assays

Piperonyl butoxide (PBO), an inhibitor of monooxygenase activity, and diethyl maleate (DEM), an inhibitor of GSTs, were used to synergise the resistant colonies, SENN-DDT and MBN-DDT. Twenty-five 2 to 3 day old mosquitoes were exposed to 4.0% PBO (SENN-DDT and MBN-DDT) or 8.0% DEM (MBN-DDT) for an hour, and then immediately exposed to insecticide (0.05% permethrin, 0.75% deltamethrin or 4% DDT) for an hour

before being returned to a holding tube. In addition, mosquitoes ($n \approx 25$) were exposed to the insecticide only (deltamethrin, permethrin or DDT) for an hour, and then as an additional control, to the synergist only (PBO or DEM) for an hour, and were then returned to holding tubes. Mortality was recorded after 24 hours. Insecticide exposure versus synergist plus insecticide exposure were analysed using a *t*-test. Three to four repeats were prepared for each insecticide/synergist assay, depending on mosquito availability.

#### RNA extractions and cDNA synthesis for microarrays

Female mosquitoes from the different colonies (SENN-base [susceptible]; SENN-DDT [resistant]; MBN-base [susceptible]; MBN-DDT [resistant]) were collected on the day of emergence and maintained on 10% sugar water. Three days later, RNA was extracted from 15 mosquitoes, representing one biological repeat. A total of three biological repeats were used in the experiment and analysis described below. RNA was extracted as described by Christian *et al*. [32].

#### Microarrays

Three independent biological repeats were performed for each colony group (SENN and MBN), and for each biological repeat, two technical repeats were performed that included dye swaps in order to compensate for dye bias. Preparation of the probes and microarrays was based on the protocol of Christian *et al*. [32], with some minor modifications based on the outcome of preliminary experiments. Briefly, amplified antisense (a) RNA was labeled by reverse transcription using Cy-dUTPs. aRNA (8 μg) was mixed with random hexamers (Invitrogen), 2 μl spike in control (Lucidea Universal ScoreCard, Amersham) and water and the mixture was incubated at 70°C for 5 minutes. The reverse transcription mix (RT Buffer, DTT, Cy3-dUTP or Cy5-dUTP, DTT, dT-NTP mix, RNAsin and Superscript® III [Invitrogen]) was added to each RNA and primer mix, and incubated at 50°C for 2.5 hours. The reaction was stopped by adding 1 M NaOH/20 mM EDTA, and incubation at 70°C for 5 minutes. The Cy-labeled cDNAs were purified using the CyScribe™ GFX™ Purification Kit (Amersham) according to manufacturer's instructions. In order to control the efficiency of the labeling and purification procedures, samples were measured on a NanoDrop using the microarray setting. Acceptable dye binding was considered to be >0.1 pmol/μl and acceptable cDNA yields were required to be >15 ng/μl. If these conditions were not met, the hybridization process was abandoned. Poly(A) was added to each cDNA mix and samples were evaporated at 37°C for an hour using an Eppendorf concentrator 5350. The cDNA was resuspended in 15.5 μl hybridization buffer (Corning) and kept in the dark until slides were ready.

During this time, the microarrays were prepared for hybridization. The Pronto!™ Universal Microarray Hybridization Kit (Corning) was used, but a 1.5x preparation of each wash solution was used, along with slightly reduced exposure times, following a series of optimization experiments. Once slides were prepared, the labeled targets were denatured by hybridization at 95°C for 5 minutes. The targets were added to each array and hybridizations were performed at 42°C for 18–20 hours. After incubation, slides were washed using the Pronto!™ Universal Hybridization Kit (1.5x solutions prepared), and dried by centrifugation at 2500 x *g* for 2 minutes.

#### Microarray scanning and data analysis

Analyses were based on those used by Christian *et al*. [32]. The arrays were scanned using the Genepix 4000B scanner (Molecular Devices, USA) where the PMT values were adjusted to give a pixel ratio of approximately 1. Spot quality and background intensities were examined and corrected using Genepix Pro 6.0 software (Axon Instruments, USA). Saturated features were recorded as such, and were excluded from analysis.

Gene expression data were analaysed using Limma version 2.12.0 (Bioconductor) [38] in R, version 2.8.0 (http://cran.r-project.org/bin/windows/base/old/2.8.0/), a command-driven program for statistical computing. Raw intensity values for each spot were calculated, and then background corrected by the method "normexp" with an offset of 50. This approach produces positive adjusted intensities and variation in log-ratios for low intensity spots are pushed toward zero (i.e. no spots are "lost" if a high background signal is measured). The corrected intensity values were transformed to log-ratios and then normalized. Control spots were used for within array normalization (i.e. normalization was based on non-differentially expressed control spots). Between array normalization was done using the "Aquantile" method where spot intensity values are transformed so that their distributions are similar between microarrays. MA-plots were viewed so that normalization could be monitored. Once analyses are complete, Limma produces a "topTable", a summary that includes the following: the gene ID, M ($\log_2$-fold change) and A ($\log_2$-average intensity) values, a moderated *t*-statistic, a *p*-value, an adjusted *p*- value, a B-statistic as well as an F-statistic (from the 'eBayes' function). Of interest to us were genes with adjusted p-values ≤ 0.05 and fold-changes ≥ 1.5. Genes in this category were considered to be statistically significant. These data have been deposited into Vectorbase (https://www.vectorbase.org).

#### Quantitative real-time PCR (qPCR)

Real-time PCR was carried out in order to validate the results of the microarray experiments. As with the microarray experiment, RNA was extracted from three

day old *An. arabiensis* females that had been supplied with 10% sugar solution. RNA was extracted from 15 mosquitoes (one biological repeat) using the TRI-Reagent® Solution (Sigma-Aldrich) and supplied methodology. A DNase treatment was included (RNase-Free DNase Set, Qiagen). Samples were quantified using a NanoDrop and then reverse transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen).

cDNA was stored at −20°C until required for PCR. For the SENN colony group, three genes were evaluated by real-time PCR (*CYP9L1* [over-transcribed], *CO1* [saturated] and *CYP4G16* [saturated]), and for the MBN group, four genes were evaluated (*CYP6P3, CYP6AK1, CYP6M2* and *TPX4,* all found to be over-transcribed in the microarray study). Primers were designed based on *An. gambiae* sequence information using either Beacon Designer™ (Premier Biosoft) or Invitrogen's free online primer design tool, OligoPerfect™ Designer. For each colony, a reference gene evaluation was conducted and the most suitable reference gene was selected from all potential candidate genes tested (ribosomal protein *S7,* ribosomal protein L19 [RPL19], the cytoskeletal protein *β-actin, GAPDH* and TATA binding protein). The data from these experiments were analysed using NormFinder (2004, Molecular Diagnostic Laboratory, Aarhus University Hospital). For the SENN colony group, gene expression was measured relative to *rsp 7,* and for the MBN colony group, gene expression was measured against *β-actin*. PCR was carried out using the Bio-Rad CFX96™ Real-Time PCR Detection System. Each reaction was set up using a total volume of 25 μl comprising 12.5 μl IQ™ SYBR super-mix (Bio-Rad), 4 μl primer (concentration optimised for each gene), 1 μl cDNA (100 ng/μl) and nuclease free water. Primer specifics, including annealing conditions and primer concentrations are described in Table 1 (SENN) and Table 2 (MBN). Standard curves were prepared by two-fold dilutions of cDNA derived from the resistant colony. Three biological repeats were evaluated, and for each biological repeat, three technical repeats were included for each reaction of interest i.e. where relative quantification was calculated. Data were analysed using the Pfaffl [39] method. Initially, PCR product for each gene of interest was sent to Macrogen for sequencing in both directions in order to confirm (over and above melt curve analysis) that the correct product was amplified in each case.

## Results

### WHO insecticide susceptibility assays

SENN-base (Table 3) was found to be resistant to permethrin (53% mortality), but susceptible to all other insecticides tested. SENN-DDT (Table 3) was resistant to DDT, propoxur, permethrin and deltamethrin, and susceptible to bendiocarb and fenitrothion. MBN-base was susceptible to all insecticides tested, while MBN-DDT was resistant to all insecticides except fenitrothion (Table 3).

**Table 1 SENN-base/SENN-DDT primer information for qPCR (F = forward, R = reverse)**

| Gene | Primer sequence | Annealing temperature | Amplicon length |
|---|---|---|---|
| *CYPL9L1* | F 5′- AGA TAA TGT ATT CTT TCG CTA TGG -3′ | 58.3°C | 188 |
| | R 5′- GCT CTT CTC GCT CTT GAA C -3′ | | |
| *CO1* | F 5′- TGC TCC TAA AAT AGA AGA AAT TCC -3′ | 58.3°C | 173 |
| | R 5′- TGC TTC CTC CTT CAT TAA CAC -3′ | | |
| *CYP4G16* | F 5′- CAG ACC GTC CAG CCA CAT TC -3′ | 58.3°C | 108 |
| | R 5′-GCG AAC GAG CAA TTA TAG GTA CTG -3′ | | |
| *rsp 7* | F 5′-TTA CTG CTG TGT ACG ATG CC-3′ | 58.3°C | 135 |
| | R 5′-GAT GGT GGT CTG CTG GTT-3′ | | |

### Microarrays and qPCR

The *An. gambiae* detox microarray was used in a cross-species hybridization study with *An. arabiensis.* As a result, a subset of arrays used for analysis were checked

**Table 2 MBN-base/MBN-DDT primer information for qPCR (F = forward, R = reverse)**

| Gene | Primer sequence | Annealing temperature | Amplicon length |
|---|---|---|---|
| *CYP6M2* | F 5′- CAT GAC ACA AAC CGA CAA GG -3′ | 60.0°C | 235 |
| | R 5′- GGT GAG GAG AGT CGA CGA AG -3′ | | |
| *CYP6AK1* | F 5′- TCA TCG AGC GAC AGT GTA CC -3′ | 58.3°C | 251 |
| | R 5′- AAA GTG TGA CCC CAG ACA GG -3′ | | |
| *CYP6P3* | F 5′- CGA TTC TTC CTG GAC ATC GT -3′ | 58.3°C | 141 |
| | R 5′- CTT GCC CAA ACT ACC GTC AT -3′ | | |
| *TPX4* | F 5′- CAG CTG ACA GAC CGA TTA AG -3′ | 58.3°C | 116 |
| | R 5′- CCG TTC GGG AAC AGT TTG TCT -3′ | | |
| *β-actin* | F 5′- ACC AAG AGC CTG AAG CAC -3′ | * | 123 |
| | R 5′- CGA GCA CGA CAC ACT ATA TAC -3′ | | |

* Annealing temperature used was the same as the target gene of interest.

**Table 3 Mortality data obtained following exposure of (A) SENN-base and SENN-DDT and (B) MBN-base and MBN-DDT to a range of insecticides, all of which belong to classes currently approved by WHO for use in vector control (*n* = number of mosquitoes exposed to insecticide)**

| Insecticide | SENN-base | | SENN-DDT | | MBN-Base | | MBN-DDT | |
|---|---|---|---|---|---|---|---|---|
| | *n* | *% mortality* | *n* | *% mortality* | *n* | *% mortality* | *n* | *% mortality* |
| DDT (4.0%) | 100 | 100 | 99 | 7.8 | 88 | 91.5 | 96 | 0 |
| Permethrin (0.75%) | 112 | 53.3 | 99 | 7.0 | 89 | 97.8 | 93 | 4 |
| Deltamethrin (0.05%) | 106 | 99.0 | 94 | 50.5 | 92 | 100 | 103 | 34 |
| Bendiocarb (0.1%) | 107 | 97.8 | 97 | 100 | 95 | 95.8 | 102 | 77.5 |
| Propoxur (0.1%) | 89 | 100 | 112 | 85.5 | 77 | 100 | 95 | 65.3 |
| Fenitrothion (1.0%) | 105 | 100 | 106 | 100 | 94 | 100 | 71 | 100 |

for probe binding success, (this was a visual assessment) and where probes did not hybridize, the probe name, and its position on the array were recorded. On average, 97.5% binding success rate was obtained in this study.

Genes that produced a fold change of ≥1.5 and an adjusted *p*-value of ≤ 0.05 after microarray analysis were considered to be differentially regulated. When SENN-DDT was compared with the relevant base colony in the microarray study, only one gene, *CYP9L1,* was found to be significantly over-transcribed (Figure 1). In the unselected equivalent, a single gene, *CYP6Z1,* was over-transcribed. In contrast, in the MBN-DDT colony 20 genes were significantly over-transcribed (Figure 2). Of these, the majority were P450 genes (50%), followed by GSTs (40%) and a small number or redox genes (one TPX and one SOD) (Table 4). Five genes consistently produced saturation on both SENN and MBN microarrays. These were *CYP4G16, CO1, GSTD5, SOD3A* and *AGM1.* The transcription of two of these genes was investigated further by real-time PCR. These genes were assessed using the colonies from Sudan.

Relative quantification was used to validate the microarray data. The expression level of *CYP9L1* in the Sudanese colony, had a fold change (FC) of 1.7 after

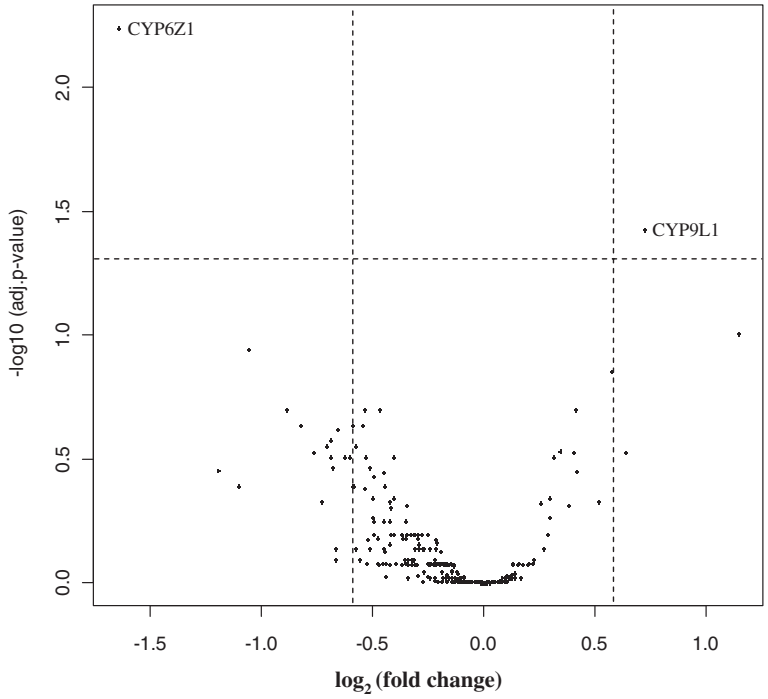



**Figure 1 The volcano plot of SENN-base and SENN-DDT microarray data.** The plot represents both statistical relevance, in the form of the *p*-value on the *y*-axis, and biological relevance in the form of the fold change on the *x*-axis. The cut-offs for significance are shown (adj. *p*-value ≤ 0.05; FC ≥ 1.5) and those genes that meet the criteria are labeled. Note that all positive fold change (FC) values represent genes over-transcribed in the resistant colony (SENN-DDT), while negative FC values represent the genes over transcribed in the susceptible colony (SENN-base).

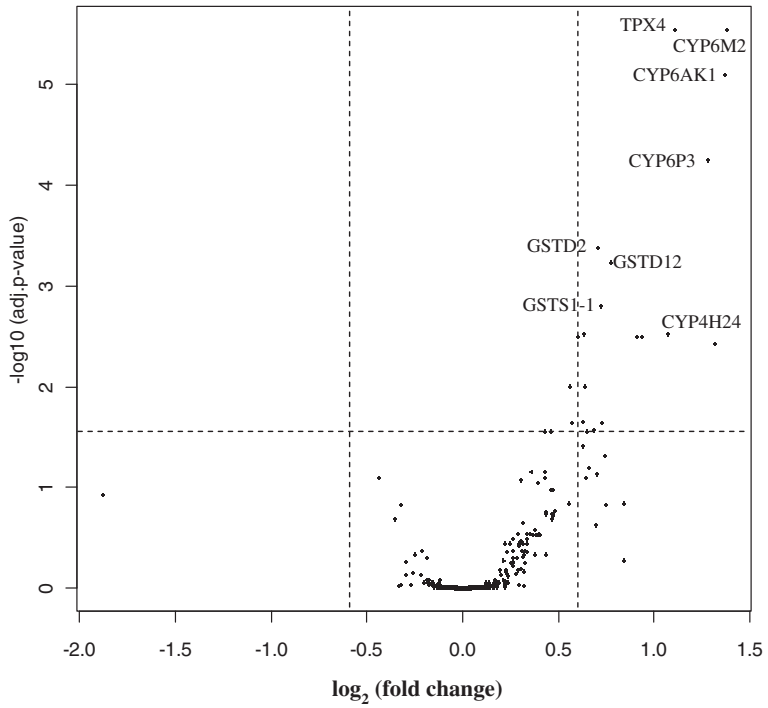



**Figure 2 The volcano plot of MBN-base and MBN-DDT microarray data.** The plot represents both statistical relevance, in the form of the *p*-value on the *y*-axis, and biological relevance in the form of the fold change on the *x*-axis. The cut-offs for significance are shown (adj. *p*-value ≤ 0.05; FC ≥ 1.5) and the top eight genes that met the criteria have been labeled. Note that all positive FC values belong to the genes that are over-transcribed in the resistant colony (MBN-DDT), while negative FC values represent those of the susceptible colony (MBN-base).

microarray analysis, and FC of 2.5 after qPCR analysis (Figure 3A). While saturated spots were flagged and not used in analyses, qPCR was also used to measure the FC difference between the susceptible and resistant Sudanese strains. In two of the five genes that were found to be saturated, the cytochrome oxidase, *CYP4G16,* produced a FC of 1.8, while *CO1,* a gene frequently associated with the resistant phenotype, produced a FC of 1.6 (Figure 3A).

In the case of South African *An. arabiensis* colony (MBN), a sample of four genes that were over-transcribed according to microarary evaluation were validated by qPCR. These genes were the four top genes based on FC and the adjusted *p*-value, namely *CYP6M2, TPX4, CYP6AK1* and *CYP6P3* (Table 4). The FCs in expression after microarray analyses were comparable to those measured by qPCR. Based on qPCR analysis, *CYP6M2* and *CYP6P3* each had fold change expression levels of more than 2 while *CYP6AK1* and *TPX4* produced fold change values of 0.9 and 1.5 respectively (Figure 3B).

### Synergist assays

The synergist assays were used to determine whether the expression of detoxification genes in each colony were in fact related to the resistance observed, or whether the phenotypes were due to the presence of *kdr.* Only one gene (a P450) was over-transcribed in SENN-DDT and so only PBO was used as a synergist in this instance. No significant difference in mortality between DDT exposure versus exposure to PBO + DDT was found (Table 5). Similarly, no significant difference between permethrin versus PBO + permethrin was observed. However, the mortality on deltamethrin versus PBO + deltamethrin was significantly different ($p = 0.0006$, $t = 7.7308$, df = 5) (Table 5). The effects of both DEM and PBO were evaluated in the MBN-DDT colony as monooxygenases and GSTs were over-transcribed in the resistant phenotype according to the microarray experiments. The synergist, PBO, had no significant impact on mosquito response to DDT or permethrin but did impact significantly on MBN-DDT response to deltamethrin ($p = 0.0004$, $t = 8.331$, df = 5). While DEM had no significant impact on DDT and permethrin resistance, a significant difference on mosquito response to deltamethrin versus DEM + deltamethrin ($p = 0.0083$, $t = 4.8596$, df = 4)) (Table 5) was observed.

## Discussion

Resistance to DDT and pyrethroids is widespread and has hampered malaria control efforts throughout Africa [2-9].

**Table 4 List of probes that were over-transcribed in SENN-DDT and MBN-DDT when compared with the susceptible equivalent**

| Gene SENN | Function | FC | Adj. *p*-value | GB accession number | Location |
|---|---|---|---|---|---|
| *CYP9L1* | Cytochrome P450 | 1.7 | 3,74E-2 | AF487781 | 3 L |
| **MBN** | | | | | |
| *CYP6M2* | Cytochrome P450 monooxygenase | 2.7 | 6.12E-6 | AY193729 | 3R |
| *TPX4* | Thioredoxin-dependent peroxidase | 2.3 | 6.12E-6 | AY745235 | 3 L |
| *CYP6AK1* | Cytochrome P450 monooxygenase | 2.6 | 2.12E-5 | AY745227 | 3 L |
| *CYP6P3* | Cytochrome P450 monooxygenase | 2.6 | 1.20E-4 | AF487534 | 2R |
| *GSTD2* | Glutathione S-transferase | 1.7 | 3.09E-4 | Z71480 | 2R |
| *GSTS1-1* | Glutathione S-transferase | 1.7 | 7.49E-4 | L07880 | 3 L |
| *GSTD12* | Glutathione S-transferase | 1.7 | 1.44E-3 | AF316638 | 2R |
| *CYP4H24* | Cytochrome P450 monooxygenase | 2.2 | 4.83E-3 | AY062206 | X |
| *GSTD3* | Glutathione S-transferase | 2.0 | 4.91E-3 | AF513638 | 2R |
| *CYP6AG2* | Cytochrome P450 monooxygenase | 2.0 | 5.24E-3 | AY745224 | 2R |
| *GSTMS3* | Glutathione S-transferase | 1.6 | 5.83E-3 | AY278448 | 3R |
| *GSTS1-2* | Glutathione S-transferase | 1.5 | 6.71E-3 | AF513639 | 3 L |
| *CYP9J5* | Cytochrome P450 monooxygenase | 2.7 | 7.73E-3 | AY748830 | 3 L |
| *CYP6P1* | Cytochrome P450 monooxygenase | 1.5 | 7.73E-3 | AY028785 | 2R |
| *SOD1* | Superoxide dismutase | 1.6 | 1.13E-2 | AY505417 | 3 L |
| *CYP6M3* | Cytochrome P450 monooxygenase | 1.8 | 1.58E-2 | AY193730 | 3R |
| *GSTU1* | Glutathione S-transferase | 1.6 | 1.58E-2 | AF515521 | X |
| *CYP12f2* | Cytochrome P450 | 1.7 | 1.83E-2 | AY176050 | 3R |
| *GSTMS1* | Glutathione S-transferase | 1.6 | 3.81E-2 | AY278446 | X |
| *CYP12F4* | Cytochrome P450 monooxygenase | 1.7 | 4.01E-2 | AY176048 | 3R |

Relevant information included is the gene function, FC, adjusted *p*-value, Genbank (GB) accession number and the chromosomal location of each gene in the *An. gambiae* genome.

Artificial insecticide resistance selection on laboratory colonies is useful as it allows one to study the resistance mechanism on a population not influenced by other environmental selection pressures. Furthermore, artificial selection in the laboratory allows us to mimic the development of insecticide resistance from repeated and continuous exposure to insecticides, a situation that wild vector populations are frequently exposed to.

The two resistant *An. arabiensis* colonies used in this study, one from South Africa and the other from Sudan, have been under DDT selection pressure in the laboratory. Bioassay data confirmed that both SENN-DDT and MBN-DDT are highly resistant to DDT. In addition to a high level of DDT resistance, the two colonies were found to be resistant to pyrethroids (deltamethrin and permethrin). The South African population showed additional resistance to carbamates, which was not present in the Sudanese colony.

The development of multiple insecticide resistance in the above mentioned colonies is supported by subsequent studies published on the same laboratory populations. The MBN colony was colonized in 2002 without detecting pyrethroid resistance in the population. However, three years later Mouatcho *et al*. [8] reported the presence of pyrethroid resistance, which was rapidly selected for (within four generations) in the laboratory and has been shown to be P450 based. The same author also showed that carbamate tolerance could be selected for from the same colonized field population. Ranson *et al*. [34] recently published a country wide study and showed that *An. arabiensis* populations from Sudan are resistant to both DDT and pyrethroids, but remained fully susceptible to carbamates and the organophosphate, fenitrothion. This supports what was observed in the SENN-DDT colony.

The fact that DDT and pyrethroid resistance in *An. gambiae* are linked has been well-documented and has been attributed to the presence of *kdr* mutations [23,25]. Specifically, *kdr* is strongly linked with DDT and permethrin resistance, and less so with deltamethrin resistance [27,40]. In *An. arabiensis,* the relationship between the presence of *kdr* mutations and resistance phenotype

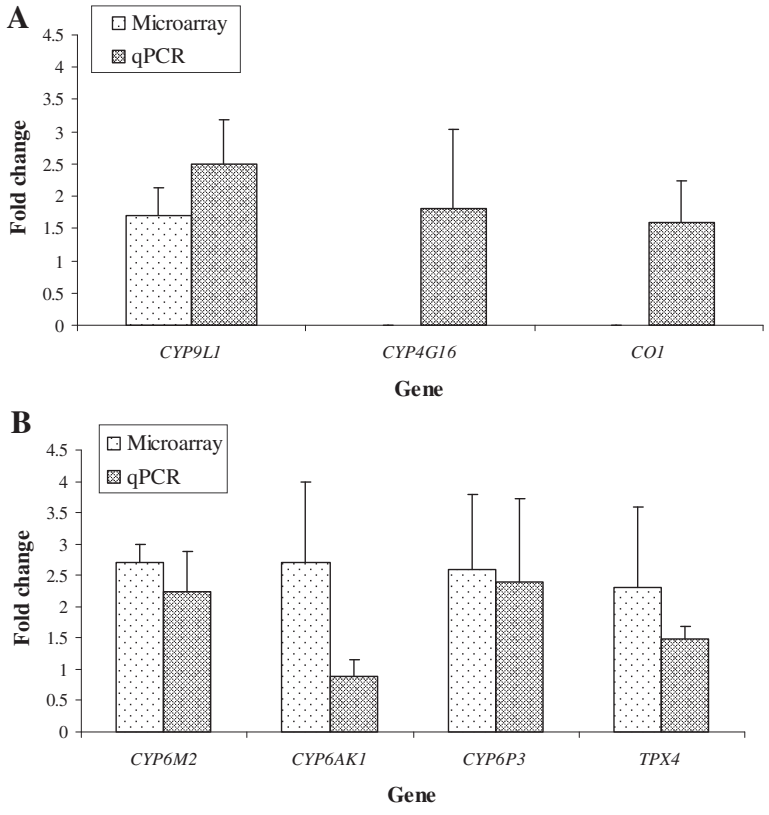



**Figure 3 A comparison of the outcome of gene expression evaluation (mean ± SD) by microarrays and by qPCR in selected genes in (A) the SENN colony group and (B) the MBN colony group.** Genes of interest were measured against the relevant reference genes.

is also complicated [2,41]. The SENN-DDT colony is fixed for the L1014F mutation. The South African *An. arabiensis* population has previously been confirmed not to carry any *kdr* mutations [7,8]. However, the continued selection pressure from exposure of MBN-DDT to DDT has resulted in this colony being fixed for the L1014F mutation. The L1014S mutation is absent from both laboratory colonies.

The detoxification enzyme profiles of the two laboratory selected DDT-resistant *An. arabiensis* strains was investigated using cross-species hybridizations of *An. arabiesnsis* genetic material with the *An. gambiae* detoxification microarray (detox chip). Of the 98% of probes that hybridized, only one gene in the SENN-DDT colony was over-transcribed. This was a cytochrome P450, *CYP9L1.* This was in contrast to the MBN colony where a similar success rate of probe hybridization was recorded, but 20 genes were highly transcribed in the resistant phenotype.

The use of the *An. gambiae* detox chip allows for the evaluation of transcription of a large number of genes simultaneously, but the criteria one uses to find significance will determine how many genes are of interest for further study. In other studies (both same- and cross-species hybridizations) the cut-off for significance in terms of fold change ranged from $>1.5$ to 2.0, and the $p$-value cut-off for significance ranged form $< 0.001$ to $<0.05$ [32,33,42-44]. Generally, where a higher fold-change was used as criteria to identify over-transcribed genes, a lower $p$-value cut-off was also used to determine significance, and vice versa. In this study, the stringency was adjusted for the wash solutions by increasing the required amount of each solution (i.e. higher than what was recommended by the supplier). The experimental conditions selected produced the best arrays, but because the experiment was based on cross-species hybridizations, we chose to use less strict criteria for identifying those genes with a significant level of differential transcription.

The action of the P450-dependent monooxygenases is one of the ways in which insects become resistant to insecticides [16]. Only one gene, *CYP9L1,* showed high expression levels in the SENN resistant phenotype and is likely to play a key role in the observed resistance to deltamethrin. The CYP9 gene family is closely related to the CYP6 family (highly expressed in the MBN resistant

**Table 5 Percentage mortality of SENN-DDT and MBN-DDT mosquitoes (females and males) to DDT and deltamethrin following exposure to synergists (*n* = number of mosquitoes tested)**

| Colony | Treatment | *n* | % Mortality (± SD) |
|---|---|---|---|
| **SENN-DDT** | PBO (4%) + DDT (4%) | 107 | 3.9 (± 4.7) |
| | DDT (4%) only | 107 | 13.0 (± 8.6) |
| | PBO (4%) only | 80 | 0 |
| | PBO (4%) + deltamethrin (0.05%) | 126 | 83.8 (± 1.3)* |
| | Deltamethrin (0.05%) only | 89 | 25.3 (± 15.6)* |
| | PBO (4%) only | 80 | 0 |
| | PBO (4%) + permethrin (0.75%) | 75 | 0 |
| | Permethrin (0.75%) only | 72 | 1.3 (± 2.3) |
| | PBO (4%) only | 79 | 2.7 (± 4.6) |
| **MBN-DDT** | PBO (4%) + DDT (4%) | 79 | 2.3 (± 2.1) |
| | DDT (4%) only | 71 | 1.3 (± 2.3) |
| | PBO (4%) only | 81 | 1.1 (± 2.0) |
| | PBO (4%) + deltamethrin (0.05%) | 78 | 70.3 (± 16.5)* |
| | Deltamethrin (0.05%) only | 97 | 2.2 (± 4.3)* |
| | PBO (4%) only | 81 | 1.1 (± 2.0) |
| | PBO (4%) + permethrin (0.75%) | 74 | 1.3 (± 2.3) |
| | Permethrin (0.75%) only | 73 | 6.7 (± 4.6) |
| | PBO (4%) only | 74 | 1.3 (± 2.3) |
| **MBN-DDT** | DEM (7%) + DDT (4%) | 80 | 1.5 (± 2.6) |
| | DDT (4%) only | 72 | 4.0 (± 4.0) |
| | DEM (7%) only | 82 | 3.5 (± 3.7) |
| | DEM (7%) + deltamethrin (0.05%) | 74 | 46.0 (± 1.7)* |
| | Deltamethrin (0.05%) only | 78 | 16.8 (± 10.2)* |
| | DEM (7%) only | 82 | 3.5 (± 3.7) |
| | DEM (7%) + permethrin (0.75%) | 75 | 1.7 (± 2.9) |
| | Permethrin (0.75%) only | 75 | 0 |
| | DEM (7%) only | 69 | 3.0 (± 2.7) |

* Indicates significant difference between insecticide versus synergist and insecticide.

phenotype) [45] and members have been linked to insecticide resistance in a number of insects [44-46]. Although not likely to be the case here, it is interesting to note that a single P450 enzyme has been implicated in resistance to DDT [47,48].

Five genes were consistently saturated when both MBN- and SENN-DDT arrays were analysed. Some of these were mainly saturated in one channel, and less so in the other, which raises the possibility that a gene is over-transcribed, but this is masked by the saturation, and might therefore be overlooked. Two of these, *CYP4G16* and *CO1,* were investigated further using qPCR and SENN-DDT genetic material. The monooxygenase, *CYP4G16* was chosen because it has previously been linked to pyrethroid tolerance in *An. arabiesnis* [31]. The cytochrome oxidase gene, *CO1,* was selected as it was over-transcribed in a microarray study on pyrethroid resistant *An. funestus* [32]. In this study, we obtained FC values of 1.8 and 1.6 for *CYP4G16* and *CO1* respectively after qPCR analysis. While these values are relatively low when compared with previously reported data, their involvement, if any, in resistance and the reason for saturation on the microarrays should be investigated further.

According to our criteria, 20 genes were differentially regulated in the resistant MBN colony and most of these genes belong to the monooxygenase and GST enzyme groups. In addition, most of the over-transcribed CYP genes belonged to the CYP6 family, which is frequently associated with insecticide resistance in insects. The top four genes were selected for qPCR validation. These were, in order of significance, *CYP6M2, TPX4, CYP6AK1* and *CYP6P3.* Recently, Munhenga and Koekemoer [49] used qPCR to assess the transcription of a range of monooxygenase genes in a pyrethroid-selected *An. arabiensis* colony from the same geographical area (KZN, South Africa). They found that *CYP6Z1* (FC = 4.7), *CYP6Z2* (FC = 1.7) and *CYP6M2* (FC = 2.2) were significantly over-transcribed. Interestingly, in our evaluation of *CYP6M2,* qPCR produced a FC of 2.2, the same level as that reported by Munhenga and Koekemoer [49], even though a different reference gene was used between the two studies.

Of the CYP genes that were over-transcribed in this study according to microarray evaluation, a number have been implicated in insecticide resistance in *An. gambiae.* Djouaka *et al*. [50] found that *CYP6P3* and *CYP6M2* were both upregulated in pyrethroid-resistant An. gambiae populations in Benin and Southern Nigeria. In permethrin-resistant *An. gambiae* from Ghana, *CYPM2*, *CYP6AK1* and *CYP6P3* were amongst the top 10 differentially expressed genes in resistant mosquitoes [30]. The authors found that the outcomes of the microarray and qPCR data were similar as was confirmed in the present study.

The GSTs also featured prominently in the enzyme profile of resistant MBN colony. The epsilon class GSTs have been specifically linked to DDT resistance in *An. gambiae* [29,51-54] and delta class GSTs to a lesser extent [52]. Furthermore, GSTs have more recently been linked to pyrethroid resistance in other insects [55,56] and so their presence in the resistance profile of MBN-DDT might be linked directly to protection against the pyrethroid, deltamethrin. Because they help to protect cells against oxidative stress, their over-expression in the MBN-DDT colony is also likely to be linked to the action of the cytochrome P450s where the GSTs are involved in secondary metabolism through the action of glutathione peroxidase [52].

A number of enzymes, namely the SODs, TPXs and GRXs, counteract the effects of reactive oxygen molecules, which are harmful to the host [57]. The SODs function by converting superoxide anions to hydrogen peroxide and oxygen [58]. In turn, the TPXs are involved in the removal of hydrogen peroxide [58]. Based on microarray experiments, we reported high levels of *TPX4* (2.3 fold) expression in the South African population of DDT selected *An. arabiensis*. This enzyme was over-transcribed in *An. arabiensis* during the spraying season of a cotton field in Cameroon [31], while *TPX1* was over-expressed in *An. gambiae,* resistant to pyrethroids, from Ghana [59]. In the MBN colony, whether the high expression of *TPX4* is related directly to the activities of the P450 enzymes (to counteract metabolic byproducts), or is a function of the insecticide resistance selection process where they are on "stand-by" to provide protection against pyrethroids, is unknown.

According to Brooke and Koekemoer [27], and references therein, the correlation between the presence of *kdr* and mosquito response to insecticide is strongest in the case of DDT, less so with permethrin, and weakest with deltamethrin. The outcome of the synergist studies performed here suggests that detoxification enzymes have no impact on DDT resistance in these strains, but are very important for protection against the pyrethroid, deltamethrin. The presence of the L1014F *kdr* mutations is likely to assist in protection against permethrin.

## Conclusions

The combination of expression data and synergist data suggests that the systems in place for insecticide resistance are extremely complex. There is a lack of understanding as to how these genes interact and support each other in the detoxification of specific insecticides and further investigation into these molecular mechanisms is needed. It is clear that the metabolic genes associated with each resistant colony are unique for that population and there was no single gene that showed an increase in transcription between South Africa and Sudan. However, a number of genes identified in this study as being over-transcribed have been flagged in other studies for their possible roles in insecticide resistance of *An. arabiensis.* It would be valuable to replicate this study in wild populations from these regions and compare the results of enzyme studies based on laboratory colonies and wild-caught mosquitoes.

#### Abbreviations

WHO: World Health Organization; DDT: Dichlorodiphenyltrichloroethane; GST: Glutathione S-transferase; P450: Cytochrome oxidase/P450; SOD: Superoxide dismutase; TPX: Thioredoxin peroxidase; *kdr*: Knockdown resistance; IRS: Indoor residual spraying; LLIN: Long-lasting insecticide treated bednet; KZN: KwaZulu-Natal.

#### Competing interests

The authors declare that they have no competing interests.

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#### Authors' contributions

LN conducted the experiments and data analyses, interpreted results, and drafted the first version of the manuscript. RC provided technical support for the duration of the study, particularly with regard to the microarrays and qPCR, and contributed to the editing of the manuscript. NC participated in the microarray data analysis and provided useful comments for the manuscript. HR contributed to revision of the manuscript. MC provided funding for the study and contributed to revision of the manuscript. LLK provided funding for the study, conceived the project, participated in coordinating the study and helped with revision of the manuscript. All authors have read and approved the final manuscript.

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# Appendix B

## 1. Preparation of synergist papers

### 1.1 Piperonyl butoxide (PBO)

Add 4.4ml PBO concentrate (90%) (Sigma-Aldrich) to 95.6ml of olive oil-acetone mixture (1:1)

Mix 0.7ml stock solution with 1.2ml acetone (carrier solution)

Spot the mixture onto a piece of Whatman (No. 1) filter paper, cut to a size of 11.5 x 15cm

Dry overnight

Place in a clean WHO bioassay tube

### 1.2 Diethyl maleate (DEM)

Add 4.1ml of DEM concentrate (97%) (Sigma-Aldrich) to 15.9ml of olive oil-acetone mixture (1:1)

Mix 0.7ml stock solution with 1.2ml acetone (carrier solution)

Spot the mixture onto a piece of Whatman (No. 1) filter paper, cut to a size of 11.5 x 15cm

Dry overnight

Place in a clean WHO bioassay tube

**2. DEPC water (0.1%)**

1ml DEPC (Affymetrix)

999ml $dH_2O$

Incubate overnight at room temperature and then autoclave for 20 minutes

**3. EDTA**

Add 186.1g of disodium ethylenediaminetetraacetate.$2H_2O$ (Saarchem, Merck) to 800ml distilled $H_2O$

Stir well using a magnetic stirrer

Adjust to pH 8 with NaOH (Saarchem, Merck)

Autoclave

**4. Tris-borate-EDTA (TBE) buffer**

**4.1 Concentrated stock solution (5x)**

54g Tris base (Merck)

27.5g Boric acid (Associated Chemical Enterprises)

20ml 0.5M EDTA (pH 8)

**4.2 Working solution (0.5x)**

0.045M Tris-borate

0.001M EDTA

**5. Luria Bertani Broth (1L)**

10g Bacto™ Tryptone (BD)

5g Bacto™ Yeast Extract (BD)

10g NaCl (Saarchem, Merck)

Adjust to 1L with distilled $H_2O$ and autoclave

**6. Luria Bertani Agar (1L)**

10g Bacto™ Tryptone (BD)

5g Bacto™ Yeast Extract (BD)

10g NaCl (Saarchem, Merck)

15g Bacto™ Agar (BD)

Adjust to 1L with distilled $H_2O$ and autoclave

When the agar cools, add ampicillin (10mg/ml)

Pour into petri dishes

**7. Ampicillin (100mg/ml)**

Add 1g ampicillin (Roche) to 10ml distilled $H_2O$

Aliquot into microcentrifuge tubes and store at -20ºC

**8. IPTG**

Add 0.12g IPTG (Promega) to 5ml distilled $H_2O$

Store at 4ºC

**9. DNase treatment using the RNase Free DNase Set (Qiagen)**

Add 3 µl DNase Buffer and 1µl DNase I to the 30µl RNA

Incubate at room temperature for 15 minutes

Stop the reaction by incubating at 70ºC for 15 minutes

Place the sample on ice for 5 minutes

Store at -70ºC

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