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Insecticide resistance charaterization, quantification, and transferal between life stages of the malaria vector Anopheles funestus giles (Diptera: Culicidae)

Wood, Oliver Richard

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INSECTICIDE RESISTANCE CHARACTERIZATION, QUANTIFICATION, AND TRANSFERAL BETWEEN LIFE STAGES OF THE MALARIA VECTOR *ANOPHELES FUNESTUS* GILES (DIPTERA: CULICIDAE)

By

Oliver Richard Wood

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

Johannesburg, 2014

DECLARATION

I, Oliver Richard Wood, declare that this thesis is my own work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

.....

Oliver Richard Wood

9 July 2014

PUBLICATIONS AND PRESENTATIONS ARISING FROM THE STUDY

PUBLICATIONS

Amenya DA, Naguran R, Lo TC, Ranson H, Spillings BL, **Wood OR**, Brooke BD, Coetzee M and Koekemoer LL. 2008. Over expression of a cytochrome P450 (CYP6P9) in a major African malaria vector, Anopheles Funestus, resistant to pyrethroids. Insect Molecular Biology **17**:19-25.

Wood OR, Hanrahan S, Coetzee M, Koekemoer LL and Brooke BD. 2010. Cuticle thickening associated with pyrethroid resistance in the major malaria vector A*nopheles funestus*. Parasites and Vectors **3**:67.

Wood OR, Spillings BL, Hunt RH, Koekemoer LL, Coetzee M, and Brooke BD. 2014. Sub-lethal pyrethroid exposure at the larval or adult life stage and selection for resistance in the major African malaria vector *Anopheles funestus* (Diptera: Culicidae). African Entomology **22**:00-00.

ORAL PRESENTATIONS

Wood OR, Brooke BD, Hunt RH, Koekemoer LL and Coetzee M. 2008. Comparison of insecticide resistance in larval and adult life-stages between three strains of the malaria *vector, Anopheles funestus*. Parasitological Society of Southern Africa 37th Annual Congress.Facutly of Veterinary Science, University of Pretoria, Pretoria, South Africa.

Wood OR, Christian RN, Hanrahan S, Koekemoer L, Coetzee M and Brooke BD. 2009. Cuticular thickening associated with pyrethroid resistance in the major malaria vector *Anopheles funestus.* 16th Congress of the Entomological Society of Southern Africa. Stellenbosch, South Africa.

POSTER PRESENTATIONS

Wood OR, Brooke BD, BL, Hunt RH, Koekemoer LL and Coetzee M. 2009. Comparison of insecticide resistance in larval and adult life-stages between three strains of the malaria vector, *Anopheles funestus*. 16th Congress of the Entomological Society of Southern Africa. Stellenbosch, South Africa.

Wood OR, Hanrahan S, Coetzee M, Koekemoer LL and Brooke BD. 2010. Cuticle Thickening associated with pyrethroid resistance in the major malaria vector *Anopheles funestus*. NICD Academic Day. Johannesburg, South Africa.

ABSTRACT

Southern African pyrethroid resistant and insecticide susceptible laboratory colonies of the malaria vector *Anopheles funestus* were investigated to further understand the phenotypic expression of pyrethroid resistance and to establish at which life stage resistance was selected. Pyrethroid resistance levels of larvae and adults were assessed at the larval and adult life stages using WHO larval and CDC bottle bioassays. Subsequent resistance levels were then assessed following targeted selections at each life stage. Tests for an association between cuticle thickness and pyrethroid resistance were based on cuticle thickness measurements using scanning electron microscope imaging of prepared tissue sections. It is concluded that pyrethroid resistance in southern African *An. funestus* is only expressed in the adult life stage, and that selection for this phenotype can only be achieved by exposing adults. It also concluded that pyrethroid tolerant or resistant females are likely to have thicker cuticles than less tolerant or susceptible females.

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NOMENCLATURE / ABBREVIATIONS

NOMENCLATURE

The below follow the same system of nomenclature: Generation number, life-stage at time of selection, current life-stage.

Larval Selections:

F_0LL - (Primary Larval Selection) Generation 0, selected as larval, currently larvae

F_0LA - Generation 0, selected as larvae, currently adults

F_1LL - Generation 1, selected as larvae, currently larvae

F_1LA - Generation 1, selected as larvae, currently adults

Adult Selections:

F_0AA - (Primary Adult Selection) Generation 0, selected as adults, currently adults

F_1AL - Generation 1, selected as adults, currently larvae

F_1AA - Generation 1, selected as adults, currently adults

ABBREVIATIONS

CDC- Centers for Disease Control and Prevention

CPD- Critical Point Drying

DDT- Dichloro-diphenyl-trichloroethane

CYP6P9- Cytochrome P450, gene family 6, sub family P, gene 9

GST- Glutathion-S-Transferase

IRS- Indoor Residual Spraying

KZN- KwaZulu-Natal

LC50- Lethal concentration which kills 50% of an exposed population

LD50- Lethal dose which kills 50% of an exposed population

SEM- Scanning Electron Microscope

TEM- Transmission Electron Microscope

WHO- World Health Organisation

CHAPTER 1- GENERAL INTRODUCTION

1.1 INTRODUCTION

The disease Malaria has the ability to inhibit a nation's economic progress by removing affected persons from the labour force (be it formal or otherwise), and by causing a strain on state resources as a consequence of additional medical treatment requirements. A further negative effect of malaria infection in children is the impact on cognitive ability, learning capacity and in some cases resulting in these children becoming dependent on state welfare.

According to the latest available World Malaria Report (2012) from the World Health Organisation (WHO) there are currently 104 countries considered to be malaria-endemic. Using current surveillance methods, it is estimated that only around 10% of the global cases of infection are detected, leading to an estimated 219 million cases of infection worldwide in 2010. Malaria is still a tremendous menace across three continents, South America, Asia and Africa as can be seen in Figure 1.1. Of the total number of cases of infection, a disproportionate number occur in Africa (WHO, 2012).

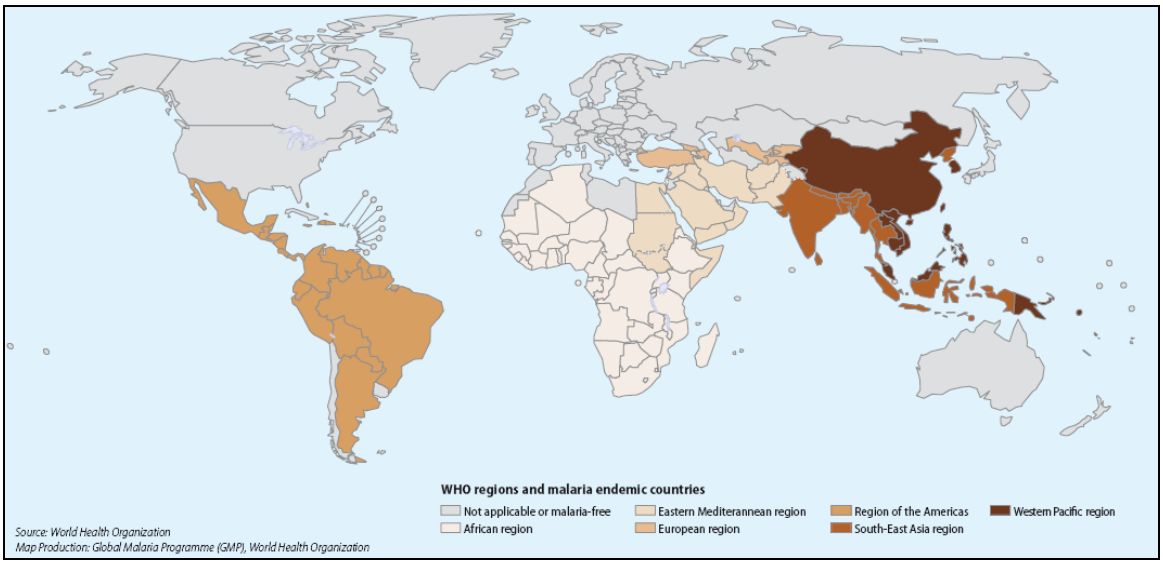

Figure 1. 1 2012 World Malaria Report map showing the World Health Organisation's Malaria zones where transmission still occurs.

1.1.1 MALARIA VECTORS IN AFRICA

In 1897, Ronald Ross proved that malaria is only transmitted by mosquitoes belonging to the *Anopheles* genus, though not all that belong to this genus transmit malaria. The four main vectors in Africa are *Anopheles funestus* Giles, *Anopheles gambiae* Giles, *Anopheles coluzzii* Coetzee & Wilkerson **sp. n.**, and *Anopheles arabiensis* Patton (Coetzee *et al*., 2013, Sinka *et al*., 2012). Their role in transmission varies between regions (Gillies & De Meillon, 1968). These three vectors fall into two taxonomic groups: *An. gambiae*, *An. coluzzii* and *An. arabiensis* belong to the *Anopheles gambiae* complex, and *An. funestus* to the *Anopheles funestus* group (Gillies & Coetzee, 1987). As this dissertation is focused mainly on *An. funestus*, the details of the *An. gambiae* complex are not discussed further here.

1.1.2 THE *ANOPHELES FUNESTUS* SPECIES GROUP

The *An. funestus* group is made up of at least nine morphologically similar species, with two currently unnamed, and a further potential for others to be detected (Coetzee & Koekemoer, 2013) This group is made up of two subgroups: Funestus and Rivulorum. Certain members of the group may be morphologically identical, while others differ only at certain life stages. The group as a whole can be considered to be primarily zoophilic, although the notable exception to this is *An. funestus* s.s. which is highly, if not entirely, anthropophilic. Other than *An. funestus s.s.*, the only other member of potential importance in malaria transmission is *An. rivulorum* Leeson (Wilkes *et al*., 1996, Kawada *et al.*, 2012). *Anopheles rivulorum* is more zoophilic than anthropophilic, so is less likely to cause a high rate of transmission. *Anopheles vaneedeni* Gillies & Coetzee has also been shown to be capable of transmitting malaria parasites, although this has only been shown under laboratory conditions (De Meillon *et al*., 1977; Coetzee & Fontenille, 2004). The remaining six members of the group are *An. leesoni* Evans, *An. parensis* Gillies, *An. fuscivenosus* Leeson, *An. aruni* Sobti, *An. brucei* Service and *An. confusus* Evans & Leeson (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987).

A recent addition to this species group is *An. funestus-like*, a newly discovered species pending a full description. This species was discovered in Malawi near the Lake Malawi shore, in the area of Karonga. The question of this new species' importance in malaria

transmission is still undetermined, but given the available evidence, it appears to be a non-vector (Spillings *et al.*, 2009, Vezenegho *et al*., 2013).

1.1.3 *ANOPHELES FUNESTUS* DESCRIPTION AND BASIC BIOLOGY

1.1.3.1 DISTRIBUTION

Anopheles funestus is distributed across most African malarious areas where suitable breeding sites are present. Its range extends from the southern Mozambique/South African border and Madagascar up to southern Sudan and west to Senegal (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987; Coetzee & Hunt, 1998, Sinka *et al*., 2012).

1.1.3.2 BREEDING SITES

Unlike *An. gambiae*, which tends to breed in temporary pools of water, *An. funestus* tends to favor more permanent and well vegetated waters such as lakes and swamps for breeding sites (Gillies & de Meillon 1968; Gillies &Coetzee, 1987), and may even include locations along slow-moving streams and rivers where there are grasses or weeds, quite often in the bends or backwaters of the river. Larvae can also be found in rice-paddies (Evans, 1938). Furthermore, in these well vegetated waters, the larvae can rapidly dive and remain submerged for protracted periods (often longer than many other species) attaching to vegetation where they are afforded shelter (Evans, 1927; personal experience).

1.1.3.3 HABITS

Anopheles funestus females are almost exclusively anthropophilic in feeding behavior. Coupled with this they are highly endophilic, entering dwellings to feed on the inhabitants. After taking a blood meal, they remain in the dwelling for several days while their eggs develop, at which point they exit in order to lay their eggs (Gillies & Coetzee, 1987).

1.1.3.4 MORPHOLOGY

Adult Female: The adult female *An. funestus* mosquito is typically described as a small species, with a wing length of 3.3 mm or less, and possessing a dark or blackish integument (Evans, 1927, Gillies and Coetzee, 1987). Size variation, possibly due to larval nutrition (Lyimo & Takken, 1993) and climatic conditions, occurs. The morphological characteristics of the *An. funestus* group are described in Gillies & Coetzee (1987). Briefly, their abdomens are devoid of lateral projecting tufts; there are 3 bands on the pale apexed palps, with the subapical pale band much narrower than the subapical dark band ; the legs are not speckled; the hind tarsi 4 and 5 are not entirely pale, with narrow and apical pale banding on the hind tarsus; the wings possess 1 pale spot on vein number 5.1; the preapical dark spot (3rd main dark area) on vein 1 does not have a pale interruption; there is at least 1 pale spot on the basal half of the costa, and a broader 3rd main dark area than the subcostal pale spot; the fringe where the dark tipped vein 6 meets it is not pale.

1.1.4 *ANOPHELES* AND MALARIA CONTROL IN SOUTH AFRICA

Anopheles funestus was eradicated from South Africa by the indoor residual spraying (IRS) of DDT that commenced in the early 1950's (Coetzee and Fontenille, 2004). In 1996, pyrethroids replaced DDT as the insecticides of choice for indoor spraying because of their relatively fast acting nature and low toxicity to mammals (Coetzee *et al*., 2013). As the Figure 1.2 illustrates, it transpired that the discontinuation of DDT was actually detrimental, as *An. funestus* in southern Mozambique had developed resistance to pyrethroids, which enabled them to expand their range across the South African border into northern KwaZulu-Natal (KZN). The incidence of malaria cases reported in South Africa increased more than six fold between 1995 and 1999 (Hargreaves *et al*., 2000, Coetzee & Hunt 2000). No previous pyrethroid resistance in *An. funestus* had been noted since Brown's report in 1986 (Brown, 1986; Coetzee & Fontenille, 2004) either due to low levels/frequencies of pyrethroid resistance, or a lack of investigation. It was only in 2000 that it was established that pyrethroid resistant *An. funestus* had re-invaded northern Kwazulu-Natal (Hargreaves *et al*., 2000, Brooke *et al*., 2001). Fortunately from a vector control point of view, this strain did not carry cross resistance to DDT, and the subsequent re-introduction of DDT in 2001 for indoor residual spraying (IRS) in traditional houses re-established control.

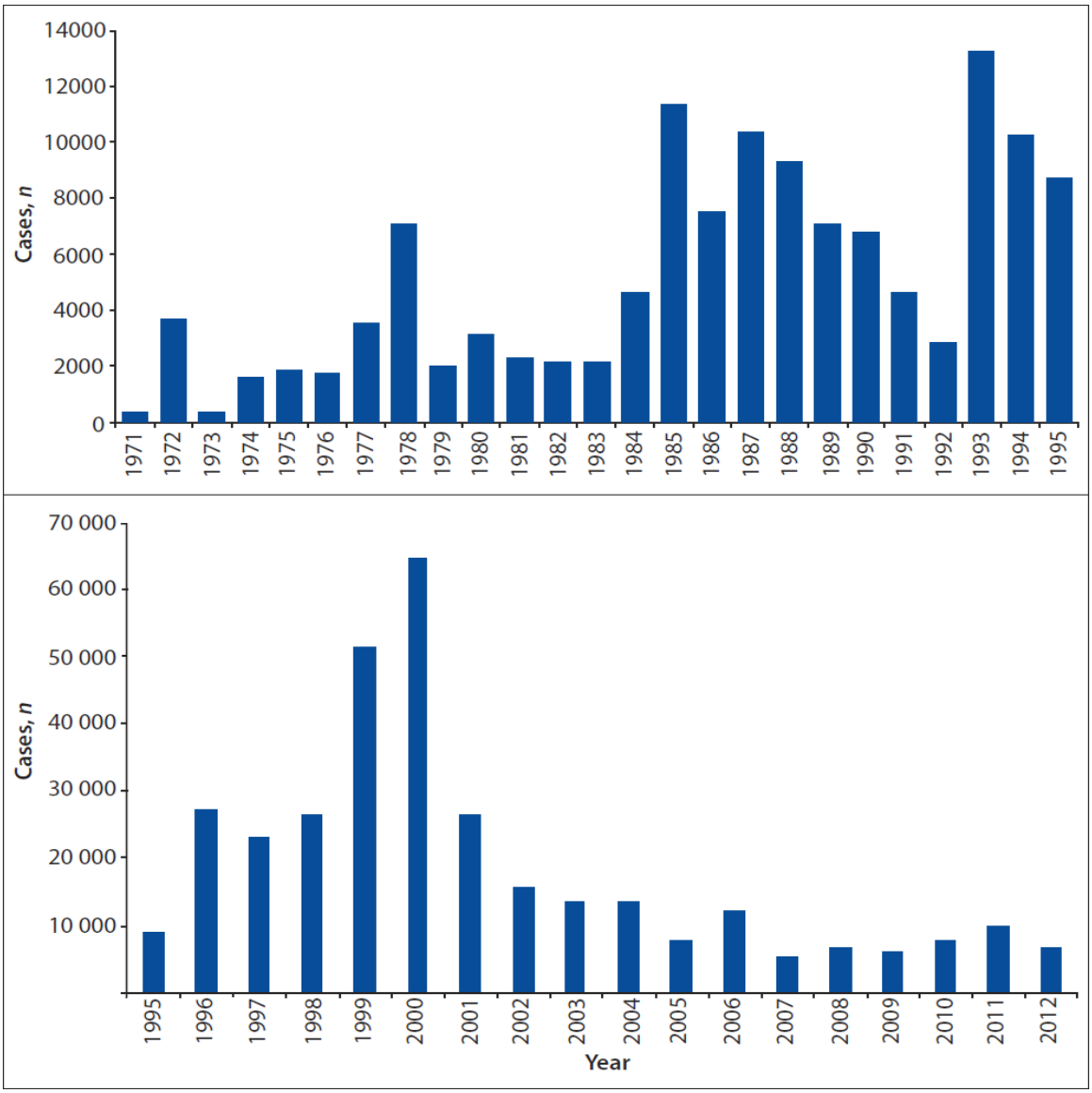

Figure 1. 2. Bar graph showing number of cases recorded annually in South Africa before and after the replacement of DDT with pyrethroids for use in indoor residual spraying (IRS) in 1996 and the effect of DDT reintroduction in 2001 (Coetzee *et al.*, 2013)

1.1.5 BACKGROUND

In 2000, the Department of Medical Entomology of the South African Institute for Medical Research, succeeded in establishing a stable *An. funestus* laboratory colony using the progeny of wild-caught *An. funestus* females from southern Mozambique (Hunt *et al.*, 2005; Brooke *et al.*, 2001). A second *An. funestus* colony was established using

specimens caught in Angola during 2003. These two colonies were named FUMOZ and FANG respectively, and show markedly different responses to pyrethroid exposure in adults (Hunt *et al*., 2005). FUMOZ is partially resistant to pyrethroids within a relatively stable range (Okoye 2008), while FANG is fully susceptible to all insecticides. A third colony, FUMOZ-R, was derived from FUMOZ and has been intensively selected for pyrethroid resistance.

Few studies have been conducted on *An. funestus* larvae investigating their resistance levels to the pyrethroid permethrin. The relatively little attention given to this vector in comparison to some of the other major vectors such as *An. gambiae,* may to some extent be attributed to a general lack of laboratory stable strains and the difficulty in locating or reaching the breeding sites discussed earlier.

In an earlier attempt to select a resistant strain from the FUMOZ colony, permethrin was added to the larval bowls in the anticipation that any emerging adults would exhibit resistance (RH Hunt, personal communication). It was found, however, that this was not a successful method for selection because the emerging adults did not show a greater resistance compared to selection at the adult level. This left the question of what an insecticide exposure at the larval stage translated to in terms of resistance in subsequent life stages.

One of the theories as to the source of selection pressure was that larvae may be exposed to insecticides from the agricultural sector leaching into water bodies where the larvae

develop. In many instances selection pressure from insecticides is believed to largely occur at the larval stage (Akogbéto et al., 2006, Chouaïou *et al*., 2008, Djouaka *et al*., 2008, David *et al*., 2010). An over expressed P450 gene was recently isolated, and analysis of the gene expression revealed highly expressed CYP6P9 only in the adult stage, but not in larvae (Amenya *et al*., 2005, Amenya *et al*., 2008). This is thought to be a possible indicator that resistance mechanisms between the adults and early developmental stages may differ.

Little comparable data on *An. funestus* larval exposures have been published, although Omer *et al.* (1980) observed that *An. stephensi* larvae with a 144-fold resistance to DDT, yielded adults with only 28-fold resistance (Omer *et al*., 1980). As in the attempt to select a resistant strain at the larval stage by Hunt (RH Hunt, personal communication), Omer *et al*. added insecticide to the point of saturation, resulting in a plateau in mortality (Omer *et al*., 1980), thus ruling out issues of differing concentrations. Gayathri and Murthy (2006) examined the accumulation of resistance over 25 generations in *An. stephensi* selected at adult and larval stages separately. It was found that adults selected for resistance at the larval stage proved to be more susceptible than adults selected at the adult stage (Gayathri & Murthy, 2006, & V Gayathri, personal communication). In an experiment, in which *Culex quinquefasciatus* were selected for malathion and permethrin resistance at different life stages, it was found that resistance to permethrin developed at a more rapid rate than to malathion in the larval exposures. The adult exposures, however, demonstrated that permethrin resistance developed slower (Selvi *et al*., 2007). This further suggests that resistance mechanisms between the adults and early developmental stages may differ.

Selvi *et al*., (2007) also investigated the levels of esterases at the various life stages. They discovered that the mean esterase expression value increased as the mosquito developed through its life stages from egg, through the various larval instars and ultimately adults with the exception of a spike at the 3rd instar (Selvi *et al.*, 2007). It is unknown if this will apply to *An. funestus.*

1.2 MECHANISMS OF INSECTICIDE RESISTANCE

In their review on the biology and evolution of toxicant resistance, Taylor and Feyereisen (1996) described three main groupings for the many possible adaptations that allow organisms to survive, what would ordinarily prove to be a, lethal dose of toxicant. The first is pharmaco-dynamically derived resistance, where there is a decreased response to a toxicant by the target. The second is pharmaco-kinetically derived resistance. This incorporates behavioural avoidance, reduced uptake, and increased detoxification, elimination and sequestration. The third mechanism they mention is that of avoidance. This does not refer to the avoidance of intoxication, which under these definitions would fall under the pharmaco-kinetic group, but rather the adoption of other metabolic pathways once a particular process is hindered to ensure the continuation of the organism's metabolism. However, such occurrences are rare, and by the time of their review there was only one known case involving nematodes. Therefore this class of toxicant resistance is not considered further here.

Merging the above definitions with more recent work, the pharmaco-dynamically derived resistance clearly represents target-site resistance, which involves target site alterations such as knock-down resistance (*kdr*) and mutations in the acetylcholinesterase genes (MACE) and GABA receptors (Hemmingway and Ranson, 2000).

Metabolic resistance refers to enzyme systems such as esterases, monooxygenases and glutathione-S-transferases (GSTs) which detoxify, sequester and aid in elimination/excretion (Hemingway and Ranson, 2000). This falls into the pharmaco-kinetically derived resistance class. Table 1.1 summarizes and illustrates the magnitude of the role played by these major effect mechanisms in conferring resistance to the various insecticide classes.

Behavioural changes such as excito-repellancy and physiological modifications such as cuticular modification both contribute towards reduced uptake of a toxicant (Ahmad *et al.*, 2006). Although they may have a bearing on improving metabolic resistance efficacy, they are not necessarily metabolic in nature and may also aid resistance mediated by reduced target site sensitivity. For the purposes of this study, it may therefore be more beneficial to consider reduced uptake and behavioural avoidance in a third group, possibly as auxiliary or indirect resistance. These are not major effect mechanisms but may contribute to the resistance phenotype by enhancing the effect of one or more primary detoxification mechanisms.

Compounding factors to all of these, which may alter the overall expression of a resistance profile, include sex-linked factors, such as esterases in the accessory glands of the internal genitalia of *An. funestus* males; as well as differential expression of genes at different ages in adult mosquitoes (WHO, 1970; Green and Hunt, 1977, Cook *et al*., 2006).

1.2.1 TOXICANT AND INSECTICIDE CLASSES

There are four main classes of insecticides currently available for use in malaria vector control. These are carbamates, organochlorines (DDT), organophosphates and pyrethroids. These four classes are all insect neurotoxins.

1.2.1.1 PYRETHROIDS AND DDT

Pyrethroids were first derived from pyrethrum flowers crushed in kerosene, which could then be used for application on surfaces, but are now readily synthesized (Casida 1980). The neural target of pyrethroids and DDT is the voltage-gated sodium channel of neurons. Intoxication results in persistent activation of the neuron (Hemingway & Ranson, 2000). Sharing of this common target site may lead to the development of cross-resistance between these two insecticide classes depending on the mode of resistance (Hemingway & Ranson, 2000).

1.2.1.2 CARBAMATES AND ORGANOPHOSPHATES

Another example of two different insecticide classes sharing the same target site is the carbamates and organophosphates (Hemingway & Ranson, 2000; Hemingway *et al.*, 2004). These two insecticide classes inhibit the cholinesterase enzyme class. Cholinesterases perform an important role in the functioning of neural systems by catalysing the neurotransmitter acetylcholine's hydrolysis in neural synapses. By inhibiting the action of the cholinesterase acetylcholinesterase, the neurotransmitter is not removed from the synaptic junctions. As the acetylcholine accumulates at the synaptic junctions, excess stimulation occurs, which is a state of toxicity (Narahashi, 2006).

1.2.2 METABOLIC RESISTANCE MECHANISMS

1.2.2.1 ESTERASES

Esterases bind to insecticides rapidly, effectively sequestering them before they reach their target site (Hemingway *et al*., 2004). The resultant slow turnover of insecticide allows for continued metabolism and excretion of the insecticide. It has also been noted that, in some instances, esterases from resistant insects have a greater ability to bind to insecticides (Hemingway & Ranson, 2000). Enhanced metabolism as a consequence of esterase mediated sequestration is a major mechanism of organophosphate and carbamate resistance (Hemingway & Ranson, 2000; IRAC, 2007). In this case the levels of esterase are usually elevated. Esterase sequestration can also play a minor role in pyrethroid resistance.

1.2.2.2 CYTOCHROME P450 (MONOOXYGENASES)

This complex enzyme superfamily comprises 107 different families (Snyder, 2000). They are highly conserved structurally and are found in most organisms (Bergé *et al*., 1998; Hemingway & Ranson, 2000). Monooxygenases are involved in metabolising harmful foreign compounds (xenobiotics). They are also involved in the degradation of juvenile and steroid moulting hormones (Bergé *et al*., 1998). Production of cytochrome P450 monooxygenases can be induced by the introduction of toxins (Bergé *et al*., 1998; Blättler *et al*., 2007). In resistant insects there is an elevation of P450's and detoxification of insecticides occurs before the insecticide can reach its target site (Taylor & Feyereisen, 1996). P450s achieve their detoxification task by inducing conformational changes in the substrate i.e. insecticide or other xenobiotic. This is achieved by binding an oxygen molecule to the insecticide (Bergé *et al*., 1998; Hemingway & Ranson, 2000). Of the P450 families, CYP4, 6 and 9 in particular, have been linked to insecticide resistance, primarily pyrethroid resistance (Feyereisen, 1999, Hemingway & Ranson, 2000, Wondji *et al*., 2007).

1.2.2.3 GLUTATHIONE S-TRANSFERASES (GST'S)

Like P450s, the multifunctional GST enzymes belong to a large family, and are involved in the detoxification of xenobiotics (Hemingway & Ranson, 2000; Enayati *et al*., 2005). This task is principally achieved by increasing the water solubility of the toxic substance, through catalysing conjugation of reduced glutathione thiol groups with electrophilic compounds, thus making them more easily excretable (Enayati *et al*., 2005).

There are generally three groups of GSTs, which are classified according to their location in the cell, although the Kappa class is located in the mitochondria of mammalian cells, and has yet to be detected in insects. The other two groups are microsomal and cytosolic (Enayati *et al*., 2005). There are three microsomal GST genes in *An. gambiae* which are bound to the membrane *(*Ranson *et al.,* 2002) however microsomal GST have not been implicated in insecticide metabolism (Enayati *et al*., 2005). As such, it is the cytosolic GSTs that appear to be responsible for insecticide and other xenobiotic detoxification and elevated levels of GST activity tend to be associated with insecticide resistant strains (Hemingway & Ranson, 2000) though different tissues in the insect may vary in GST activity, such as midgut and fat body, relating to their possible function and may play different roles other than direct insecticidal protection, although deoxidative stress protection may also play a supporting role in resisting the effects of insecticides (Enayati *et al*., 2005). This is suggested as a possible role GST's may play in relation to pyrethroid resistance, as they have yet to be shown to directly metabolize pyrethroids (Vontas *et al*., 2001).

GST's are primarily associated with DDT resistance (Enayati *et al*., 2005). The mode of action by which this is achieved is through dehydrochlorination, whereby a hydrogen atom is abstracted from the DDT . This results in the chlorine being eliminated from the molecule leaving behind DDE. This process is catalysed by GSTs. In addition to DDT resistance, they have also been associated with various cases of organophosphate resistance via either O-dealkylation or O-dearylation, where the gluathion conjugates

with the alkyl portions of the molecule or reacts with leaving group respectively (Enayati *et al*., 2005).

1.2.3 TARGET SITE RESISTANCE

1.2.3.1 KNOCK DOWN RESISTANCE (*kdr*)

Knock down resistance (*kdr*) describes the resistance phenotype that results from reduced target site sensitivity to the binding of insecticide (Martinez-Torres *et al*., 1998; Hemingway & Ranson., 2000). The *kdr* phenotypes are the products of non-silent point mutations that alter the conformation of the sodium ion channel. This conformation change must simultaneously reduce affinity for insecticide binding without interfering with the natural functioning of the target site. This places severe limitations on the number of amino acid substitutions that can produce both of these effects. The *kdr* mutation found in pyrethroid resistant *An. gambiae* from West Africa has a phenylalanine substituted for a leucine at position 1014 in the second domain of the S6 transmembrane segment of the sodium channel gene (Martinez-Torres *et al*., 1998; Hemingway & Ranson, 2000; Hemingway *et al*., 2004). In East Africa an alternative substitution at position 1014 was detected, where the same leucine was replaced with a serine (Ranson *et al.*, 2000). Mutations in the sodium channel target site confer resistance to both DDT and pyrethroids.

1.2.3.2 GABA RECEPTORS

Target site resistance can also be mediated by mutational changes in gamma-aminobutyric acid (GABA) receptors. GABA receptors are inhibitory neurotransmission chloride-ion channels found in the insect central nervous system and neuromuscular junctions. Targeting of the GABA receptors is principally achieved by cyclodiene insecticides such as dieldrin, but pyrethroids have also been implicated (Hemingway & Ranson, 2000) as well the phenyl pyrazole insecticide, fipronil (Hemingway *et al*., 2004). Target site modification arising from point-mutations (alanine-to-serine) can occur in GABA receptors, imparting resistance to dieldrin and fipronil (Hemingway & Ranson, 2000; Brooke *et al.*, 2000; Du *et al*., 2005). A rarer substitution is alanine-to-glycine (Hemingway *et al.*, 2004).

1.2.3.3 ACETYLCHOLINESTERASE

Acetylcholinesterase (AChE) is responsible for the hydrolysis of the excitory neurotransmitter acetylcholine in the synaptic cleft (Hemingway & Ranson, 2000). By targeting acetylcholinesterase, organophosphates and carbamates inhibit the hydrolysis of the neurotransmitter. This results in a permanently excited neuron. Carbamate/organophosphate-resistant insects may exhibit mutational alterations in AChE, which leads to decreased sensitivity to induced inhibition (Hemingway & Ranson, 2000).

Table 1. 1. Illustration of the relative roles played by various insect mechanisms conferring resistance to various insecticide classes (IRAC, 2007)

	Metabolic			Target-site		
	Esterases	**Monoxygenases**	**GST**	***kdr***	**AChE**	**GABA**
Pyrethroids	Small	Large		Large		
DDT		Small	Large	Large		
Carbamates	Small				Large	
Organophosphates	Large	Small			Large	
Cyclodienes						Large

1.3 AIM

Based on the hypothesis of similarity in underlying resistance mechanism/s between larvae and adults of laboratory reared *Anopheles funestus*. The aim of this project was to investigate how insecticide selection at particular life stages affects the expression of the resistance phenotype in subsequent life stages.

1.4 OBJECTIVES

Specific objectives were:

1. Baseline studies: Determine the levels of permethrin induced baseline LC_{50} and LD_{50} (Lethal Concentration/Dose resulting in 50% mortality) for *An. funestus* larvae and adults respectively.

2. Larval selection: Determine the F1 progeny LC_{50} values for larvae and test for resistance in adult stages following selection at the larval stage.
3. Adult selection: Determine the F1 progeny LC_{50} values for larvae and test for resistance in adult stages following selection at the adult stage.
4. Assess cuticle thickness in association with insecticide resistance/tolerance in adult *An. funestus.*

CHAPTER 2- MATERIALS AND METHODS

2.1 MOSQUITO STRAINS

Three *An. funestus* strains were used. These colonies are housed in the Botha De Meillon Insectary at the National Institute for Communicable Diseases in Johannesburg, South Africa. This insectary is maintained at 25-27°C and 70-90% relative humidity.

The fully insecticide susceptible *An. funestus* FANG strain originates from southern Angola. This colony was established in 2003 using the progeny of wild caught females. The *An. funestus* FUMOZ base colony, established in 2000 using the progeny of wild caught females from southern Mozambique, carries pyrethroid (permethrin) resistance at a phenotypic frequency of approximately 60% in each generation without selection of any kind (Okoye, 2008). The *An. funestus* FUMOZ-R colony has been intensively selected for permethrin resistance over successive generations. This colony is almost fixed for permethrin resistance (Hunt *et al*., 2005).

2.2 DETERMINATION OF THE BASELINE LEVELS OF RESISTANCE TO PERMETHRIN IN THE LARVAL AND ADULT LIFE STAGES OF LABORATORY REARED *ANOPHELES FUNESTUS*

Larvae and adults from each of the three colonies were exposed to a series of permethrin concentrations in order to determine LC_{50} and LD_{50} values (Lethal Concentration/ Dose

inducing 50% mortality) using a log probit transformation. Quantification of susceptibility/resistance in this way enabled direct comparisons between samples as well as the determination of appropriate insecticide concentrations for subsequent resistance selection.

2.2.1 LARVAE

The larval insecticide susceptibility level bioassays were based on the guidelines given by WHO (1998). In order to determine LC50's for larvae from each of the *An. funestus* strains, 4th instar larvae were gently scooped from the parent colony rearing bowls using a clean (uncontaminated) fine meshed plastic tea strainer. While still in the strainer, distilled water was gently passed over the larvae twice to wash off any organic matter and fouled water carried over from the rearing bowl. Following each washing the convex side of the strainer was gently blotted with tissue paper to draw off the rinse water. The strainer was then inverted over a small bowl of distilled water and the larvae were gently washed into the new bowl.

Using a pipette, 25 larvae were transferred to each of a series of polystyrene cups containing 80 ml distilled water. The larvae were left to acclimatize for at least one hour. One ml of acetone solution containing the desired concentration of permethrin was added to each cup, which was then topped up to 100 ml with distilled water. The water in each cup was gently agitated to ensure a homogenous insecticide solution. The concentration range chosen: 1X 10^{-6}; 1X 10^{-5}; 1X 10^{-4}; 1X 10^{-3}; 5X 10^{-3}; 1X 10^{-2} μg was found to be

sufficient to generally achieve both 0% and 100% mortality for each of the *An. funestus* colonies. Exposure to each concentration was replicated a minimum of eight times per colony. Controls were exposure of larvae in cups containing 99 ml of water with the addition of 1 ml of acetone water only.

Following the 24 hour insecticide exposure, each cup was gently drained through a clean (decontaminated) strainer. The larvae in the strainer were gently washed twice with distilled water and excess water was removed by gently blotting the strainer with tissue paper. The larvae were then gently washed into recovery cups containing about 80 ml fresh distilled water, and topped up to 100 ml.

The WHO recommended volume of water for the cups is a guideline based on surface area to depth relationship, to take into account the various volumes of disposable cups available. Certain depth and surface area need to be met to ensure that the larvae are not agitated due to over crowding, and the depth is able to serve as a cut-off limit for any larvae too adversely affected by the exposure to rise to the surface and obtain air.

Mortality was scored following a two-hour recovery period. There are certain complications when scoring larval mortality. Larvae may die whilst resting on the water surface, and do not necessarily sink. Further, live *An. funestus* larvae can remain submerged for long periods, and thus potentially be confused with dead larvae (personal observation, Evans, 1927). It was found in this study that the most reliable method was to use the shaft of a dissecting needle to gently submerge any larvae floating on the

surface. The needle was then used to tickle the ventral side of any larvae that remained submerged. If a response of intense swimming or surfacing was not produced, the needle was then used to probe the siphon, though often it was found the dorsal tickling produced better results. Should a response still not be elicited, the larva was preliminarily counted as moribund or dead.

Mortality was scored by calculating the proportion of larvae that were moribund and unresponsive. After the initial assessment of mortality, a further ten minutes of continual monitoring followed to ensure accuracy, as some larvae that initially appeared moribund subsequently rose to the surface either in response to the needle stimulus or a need for air.

2.2.2 ADULTS

In order to determine the LD50's for adults from each of the *An. funestus* strains, Centers for Disease Control and Prevention (CDC) bottle assays were conducted, where permethrin coated 250 ml bottles were prepared at least 48 hours before their required use, as described by Brogdon & McAllister (1998). Care was taken to store the coated bottles in a dark location to avoid Ultra-Violet degradation of the insecticide.

Prior to insecticide treatment, all bottles were thoroughly washed by scrubbing in hot water and Extran detergent. The bottles were then repeatedly rinsed first with clean hot tap water. The tap water was then rinsed off twice with 100% ethanol. The ethanol was then rinsed off twice with distilled water. The bottles were then allowed ample time to

thoroughly dry before coating. A few hours before coating with a permethrin-acetone mixture, the bottles were placed on a bench to ensure that the glass reached room temperature.

The bottles were prepared by carefully pippetting 1000 µl of permethrin-acetone solution at the desired concentration into the bottle. The range of concentrations used was 1; 5; 25; 50; 100 and 250 µg per bottle. Care was taken to rotate each bottle whilst pippetting ensuring the inside of the neck and shoulders were also evenly coated. The bottle was gently shaken in a horizontal movement while the bottle was upright to ensure the base of the bottle was properly coated. Each bottle was then placed horizontally on a mechanical roller (Fig 2.1) and set to rotate at 30 revolutions per minute. The bottles were rolled for at least 20 minutes to ensure complete evaporation of the acetone. After rolling, a piece of netting was used to cap the bottle and held in place by a rubber band. A slit was made in the net cap large enough to allow the insertion of an aspirator tube. When the tube was not in place, the slit was plugged with cotton wool. Control bottles were prepared using 1000 µl acetone only per bottle. Each bottle was used twice, where upon it was smashed and disposed of by incineration.

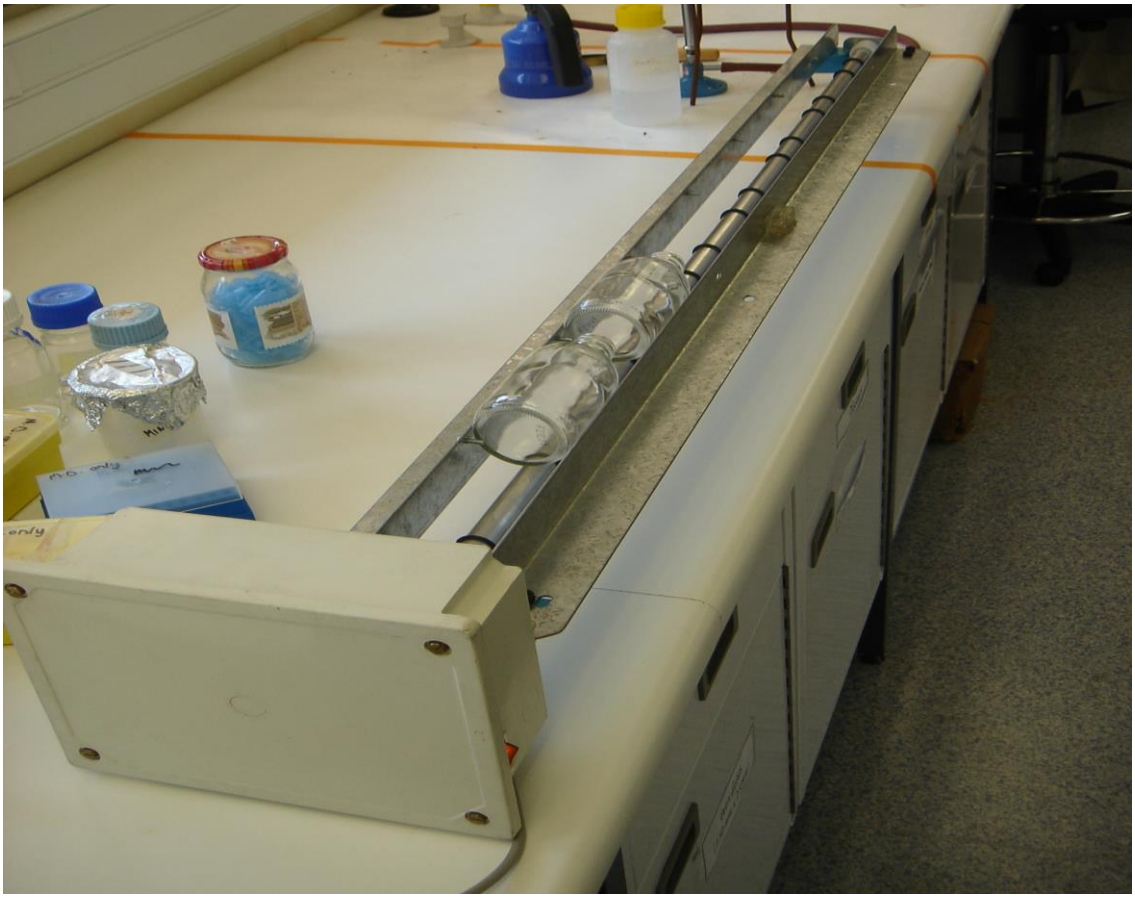

Figure 2. 1. The mechanical bottle roller, allowing for an even and consistent internal coating of bottles with insecticide.

To conduct the bioassays, 25 females were gently aspirated into each coated bottle. Exposure to each dose or control was replicated a minimum of 5 times per colony. A limiting factor in the number of replicates was the emergence rate of females from the pupae, as there were not always sufficient females within the same age range to conduct a statistically sound exposure of 25 females (a bare minimum of 20), and still have a control exposure. Exposures were conducted for 1 hour following which all mosquitoes were removed and placed into appropriately labelled cups. Each cup was covered with a piece of netting with a slit large enough to allow the insertion of an aspirator. A wad of cotton wool soaked in a 10% sucrose solution was placed on top of each cup to allow

survivors to feed. Final mortalities were scored 24 hours post exposure and the LD50's for each colony were calculated as described earlier in this section.

2.3 SELECTIONS

A summary of the nomenclature used to denote the various and possible selection and life stage combinations is provided in Table 2.1.

2.3.1 SELECTION FOR PERMETHRIN RESISTANCE AT THE LARVAL STAGE

A large number of 4th instar larvae (approximately 1000-2000) were placed in a wide container containing a small amount of distilled water. (When numbers larger than 2000 were required for selection, this was simply replicated to avoid crowding of the larvae.) Before selection began an appropriate number of larvae were set aside for determining their resistance level against the LC50 previously determined for each colony. These were denoted as F_0LL (Generation number, life-stage at time of selection, current life-stage). Table 2.1 shows the experimental design, illustrating the nomenclature derived for the various and possible selection stage and life stage combinations assessed.

Each large container was treated with a permethrin solution at the required LC50 for each colony. After a 24 hour exposure period, all larvae from each treatment were removed by straining through gauze netting, washed and transferred to a clean bowl with fresh

distilled water. The survivors were reared through and emerging adults were pooled into a cage containing a vial of cotton wool soaked in a 10% sucrose solution. Samples used for determining the permethrin LD50 were denoted F_0LA. The remainder of each cohort were given human blood meals and encouraged to lay eggs. The hatchlings from these eggs were labelled as F_1LL. The F_1LL's were reared to 4th instar where once again samples were drawn from the cohort for assessing their level of resistance to their levels compared with their respective baseline LC50. The remainder of the cohort was reared to adulthood. These were denoted F_1LA and were used to determine their resistance level to their respective baseline LD50.

Table 2. 1. A summary showing experimental outline after baseline resistance levels had been determined, and the nomenclature used at each life stage. The nomenclature pattern starts with the current generation (F#), followed by the life stage at which selection took place (L=Larval; A=Adult), followed by the current life stage (L=Larval; A=Adult), at which the permethrin resistance level was assessed.

			Selection point	Change of Life Stage		
				1st	2nd	3rd
Selection Line	**Larval**	Resistance Level Determination Bioassay	WHO Larval Bioassay	CDC Bottle Bioassay	WHO Larval Bioassay	CDC Bottle Bioassay
		Generation	Primary	Primary	1st	1st
		Nomenclature	F0LL	F0LA	F1LL	F1LA
	Adult	Nomenclature	F0AA	F1AL	F1AA	NA
		Generation	Primary	1st	1st	NA
		Resistance Level Determination Bioassay	CDC Bottle Bioassay	WHO Larval Bioassay	CDC Bottle Bioassay	NA

2.3.2 SELECTION FOR PERMETHRIN RESISTANCE AT THE ADULT STAGE

Exposure of the adults to permethrin was performed using bottles coated internally with the required LD50. A large number of 2 to 3 day old adults (both male and female) were drawn from the same cohort of each colony and placed in a cage where only sugar water was available. Samples of males and females (25 per bottle) were then drawn for at least 5 exposure replicates. All exposures were conducted as previously described. This life stage was denoted F_0AA. Following exposure all mosquitoes were removed and placed in cups to obtain the final mortalities 24 hours post exposure. Survivors were then removed from their recovery cups and placed in a cage.

The survivors of the selection were given human blood meals, and encouraged to lay as many eggs as possible. The resulting hatchlings were designated F_1AL (1st generation, selected at the adult life-stage, currently in the larval life-stage). These were reared to 4th instar where samples were drawn from the cohort. These were assayed against their appropriate LC50 to determine their resistance level The rest of the cohort was reared to adulthood and denoted F_1AA. Those remaining were used in bottle assays against their appropriate LD50 to determine their resistance levels.

CHAPTER 3- SELECTIONS AND BIOASSAY RESULTS

3.1 RESULTS

3.1.1 LARVAL AND ADULT BASELINE RESISTANCE LEVEL DETERMINATION

The LC50's derived from pyrethroid exposed larvae and the LD50's derived from pyrethroid exposed adult females are given in Figure 3.1. There was no significant variation in LC50 between FANG, FUMOZ and FUMOZ-R larvae (ANOVA: df=2; F=1.93; P=0.17). However, the variation in LD50 between adult females from each colony was highly significant (ANOVA: df=2; F=68.1; P=<0.01) with FANG giving the lowest LD50 and FUMOZ-R the highest. There was a 76.1 fold increase in the FUMOZ-R LD50 over FANG.

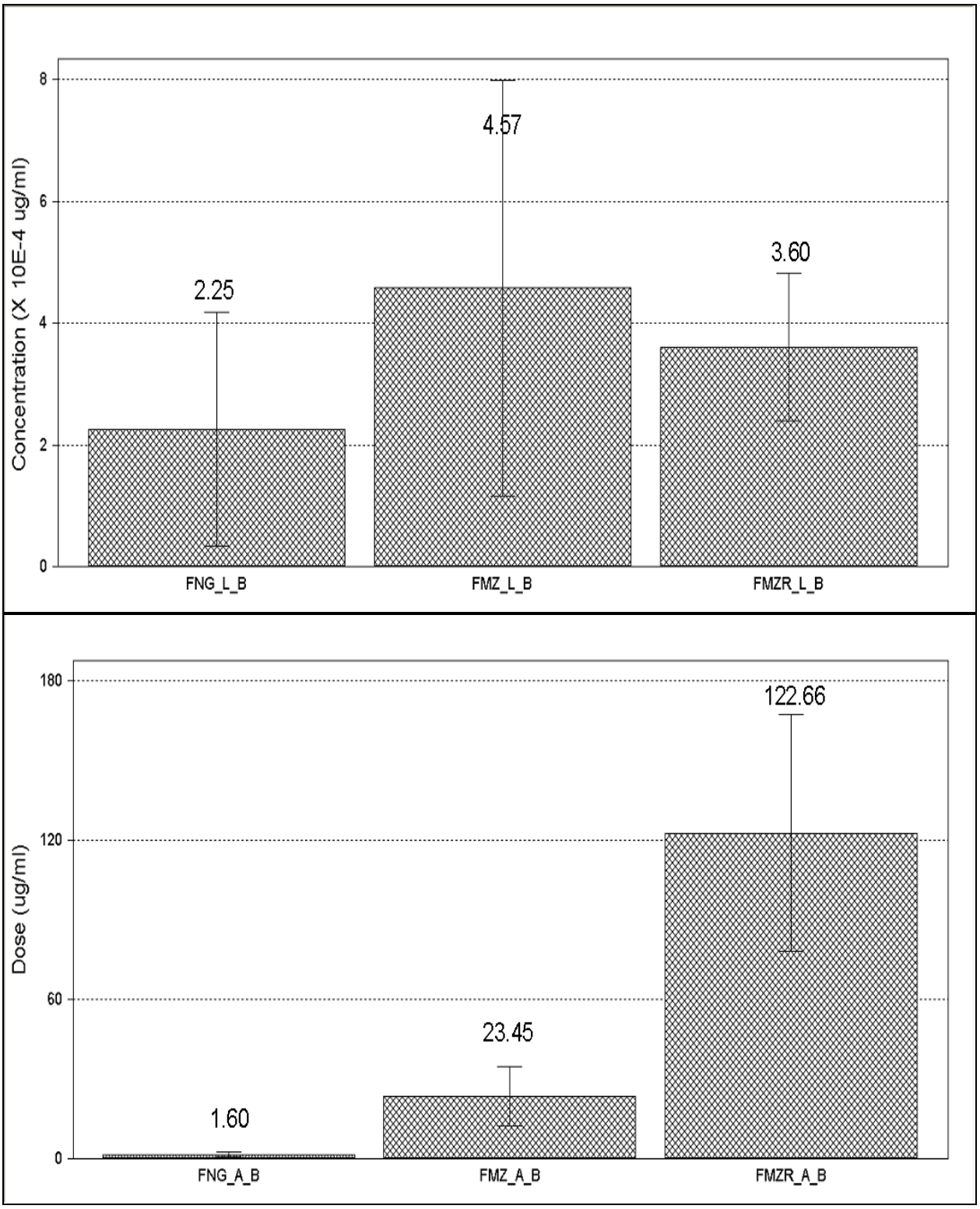

Figure 3. 1. Mean lethal permethrin concentrations inducing 50% mortality (LC50) for larvae (L-B, top) and mean lethal dosages inducing 50% mortality (LD50) for adult females (A-B, bottom) from the *Anopheles funestus* FANG (FNG), FUMOZ (FMZ) and FUMOZ-R (FMZR) laboratory colonies.

3.1.2 SELECTIONS

Assessments of permethrin resistance following selection for resistance at the larval and adult stages are summarised in Figure 3.2. Selection for resistance at the larval stage induced a significantly increased permethrin tolerance in larvae of the subsequent generation in FANG (ANOVA: df=22; F=85.3; P=<0.05) and FUMOZ-R (ANOVA: df=21; F=43.3; P<0.05). This trend was not apparent in FUMOZ (ANOVA: df=19; F=8.7; P=>0.05). This same selection significantly decreased permethrin tolerance in adults of the subsequent generation in FANG and FUMOZ (ANOVA - FANG: df=11; F=12.7; P<0.05; ANOVA - FUMOZ: df=14; F=17.5; P<0.05) but not in FUMOZ-R (ANOVA: df=10; F=1.62; P=0.23).

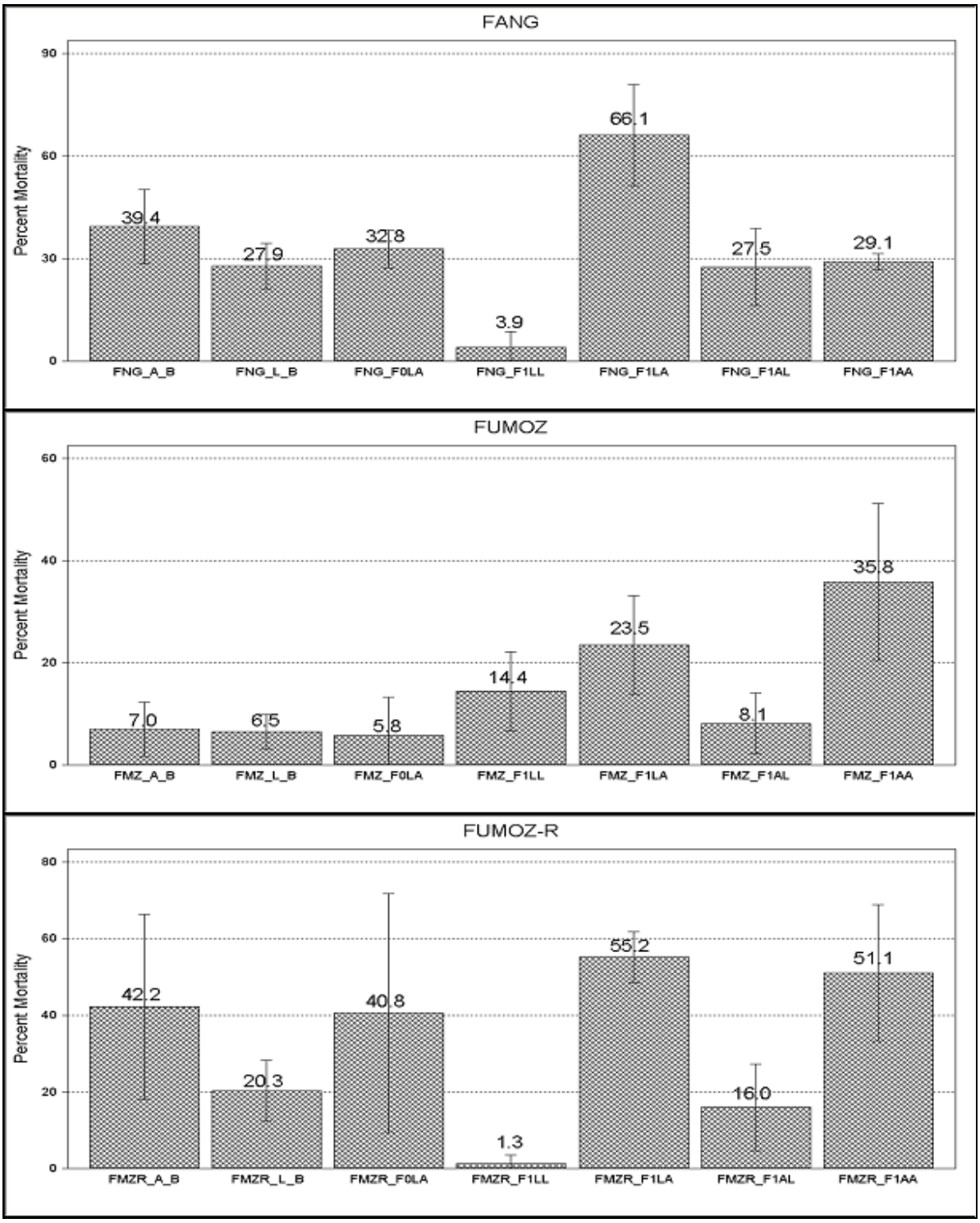

Figure 3. 2 The mean percentage mortalities induced upon various life stages following the different selections by their respective LC/LD50's for the three laboratory stable Anopheles funestus colonies FANG (FNG), FUMOZ (FMZ) and FUMOZ-R (FMZR).

Selection for resistance at the adult stage did not affect permethrin tolerance in the larvae of subsequent generations in any of the colonies (ANOVA-FANG: df=10; F=3.16; P=0.11>0.05); (ANOVA-FUMOZ: df=15; F=0.16; P=0.69); (ANOVA-FUMOZ-R: df=12; F=7.17; P>0.05). It also did not affect permethrin tolerance in the adults of subsequent generations in FANG (ANOVA: df=9; F=3.36; P=0.1) and FUMOZ-R (ANOVA: df=9; F=0.44; P=0.53), whilst permethrin tolerance in FUMOZ significantly decreased following selection in adults of the previous generation (ANOVA: df=12; F=24.7; P <0.05).

3.2 DISCUSSION

The highly significant variation in permethrin tolerance between adults of FANG, FUMOZ and FUMOZ-R is not apparent in the larvae of each strain. Despite the measurable occurrence of permethrin resistance in adult FUMOZ and FUMOZ-R compared to the insecticide susceptible FANG, the larval response to permethrin exposure between all three colonies is similar, showing that the permethrin resistance phenotype in FUMOZ and FUMOZ-R is only expressed in the adult stage.

Based on the assessments of permethrin resistance following selection at the larval and adult stages, it was not possible to enhance adult resistance/tolerance by one round of selection in any of the *An. funestus* colonies. This may have occurred because FANG does not carry selectable permethrin resistance alleles whilst permethrin resistance in FUMOZ-R is likely fixed, precluding the possibility of enhancing resistance by selection of the adults of this colony any further. The measurable decrease in permethrin resistance

in FUMOZ adults following selection in adults of the previous generation is an anomaly that cannot be readily explained. The data for FANG and FUMOZ-R showed that larval tolerance to permethrin can be enhanced by selection at the larval stage of the previous generation, although this effect was not apparent in FUMOZ. Most importantly, these data showed that selection for permethrin resistance/tolerance at the larval stage reduced permethrin tolerance in adults of the subsequent generation in FANG and FUMOZ.

The absence of measurable permethrin resistance phenotypes in FUMOZ and FUMOZ-R larvae despite the clear occurrence of the resistance phenotype in adults from the same colonies is in accord with the molecular data of Amenya *et al*. (2008) which showed that the pyrethroid resistance associated *CYP6P9* is only up-regulated in FUMOZ-R adults. It thus follows that selection for pyrethroid resistance in FUMOZ and FUMOZ-R, and thereby the wild *An. funestus* population from which they were derived, most likely occurred at the adult stage as opposed to the larval stage. This is important because insecticide resistance in vector populations often occurs at the larval stage as a consequence of the leaching of insecticide into their breeding sites following insecticide application for agricultural pest control purposes (van der Werf 1998). Yet the data presented here for southern African *An. funestus* suggest that permethrin selection at the larval stage is likely to suppress the occurrence of resistance in adults. Therefore, the occurrence of insecticide resistance in southern African *An. funestus* was likely selected as a consequence of exposure of adult mosquitoes to insecticides.

CHAPTER- 4 CUTICLE THICKNESS INVESTIGATIONS

4.1 INTRODUCTION

In addition to reduced target site sensitivity and metabolic detoxification, insecticide resistance may result or be assisted through another mode of action, which ultimately results in a reduced uptake of the insecticide. This may be achieved by means of modification to either behaviour or the organism's physiology, or both. The excito-repellent effect induced by some insecticides is an example of a mode of this behaviour. Other modes of resistance are physiological and structural attributes that inhibit the rate at which insecticides interact with their neuronal targets. A candidate mechanism is cuticle thickening.

Thickened cuticles are likely to reduce the rate at which insecticides penetrate through the cuticle and into the haemocoel. The potential effect of this thickening is that a smaller dose of insecticide inoculates the internal cavity of the mosquito in any given period, thus facilitating the effectiveness of metabolic insecticide detoxification.

The development of resistance to pyrethroid and carbamate insecticides in southern African *An. funestus* populations (Hargreaves *et al*, 2000; Casimiro *et al*., 2006) is primarily based on monooxygenase detoxification (Brooke *et al*., 2001; Amenya *et al*., 2008; Wondji *et al*., 2009). Yet it is conceivable that factors other than the primary detoxification mechanism are involved in the construction of the resistance phenotype.

In a preliminary micro-array analysis comparing gene regulation between pyrethroid resistant and susceptible *An. funestus*, upregulated expression of a gene associated with cuticle deposition (JV2) was observed amongst several other genes (Christian, unpublished data). This provided the impetus to compare cuticle thicknesses between laboratory reared *An. funestus* samples characterised by their responses to pyrethroid intoxication. A direct measure of association between these two phenotypes (response to insecticide intoxication and cuticle thickness) provides a critical platform for establishing whether they share a causal relationship, as well as for predicting the potential biological and epidemiological implications of such a relationship.

Given that the adult would not ingest the insecticide from the wall like feeding larvae would ingest it when immersed in insecticide treated water, the following investigations were not applicable to larvae.

4.2 PRELIMINARY TRIALS

4.2.1 MATERIALS AND METHODS

In order to establish the most effective and easily repeatable microscopic technique with which to conduct the cuticle thickness investigations, preliminary tests with light microscopy, transmission and scanning electron microscopy (TEM and SEM respectively) were performed.

4.2.1.1 SPECIMEN PREPARATION

4.2.1.1.1 LIGHT AND TEM MICROSCOPY

The handling and preparation of the specimens for both the light and transmission electron microscopy were the same up until the point of staining for viewing following sectioning, using Humason (1967) as a guideline.

A sample of 2-3 day old *An. funestus* was drawn from the FUMOZ laboratory colony. FUMOZ carries resistance to the pyrethroid insecticide permethrin at comparatively stable frequencies ranging between 5% and 30% mortalities (Okoye, 2008), as measured using standard insecticide exposure assays against adults (WHO, 1998). All the specimens, both the survivors and dead following assays, were killed by placing them in the fridge so that any possible further cuticle deposition could not occur.

4.2.1.1.1.1 FIXATION

The specimens were removed from the fridge and washed three times in a sodium phosphate buffer (pH 7.2) to remove dust and other artefacts. Any loose scales were also be removed by this procedure, facilitating cleaner sections.

The mosquitoes were dissected into head, thorax and abdomen in 4% gluteraldehyde, and were then moved to their respective containers where they were incubated at 4°C for 24 hours. Following this fixation period, the gluteraldehyde was drained off and the specimens were washed three times in sodium phosphate buffer (pH 7.2). They were then

removed to 1% osmiumtetroxide for post-fixation for a period of one hour. The osmiumtetroxide was then drained before the specimens were washed a further three times with sodium phosphate buffer.

The specimens were passed along a graded alcohol dehydration series:

10%; 25%; 50%; 75%; 100%; 100%

Each of the first four dehydration steps was for a lasted at least 1 hour. The specimens were then soaked in absolute alcohol for at least two hours before being moved to a final change of absolute alcohol (which had been kept under a molecular sieve to remove any possible moisture) and left to incubate overnight. While the specimens were dehydrating overnight, the components of the embedding resin (Spurr's) were removed from the 4°C fridge and allowed to reach room temperature gradually. This was done to minimize the chance of condensation droplets contaminating the resin during mixing.

The Spurr's resin comprised: an epoxy equivalent, vinylcyclohexene dioxide (VDC/ ERL 4221), a cycloaliphatic diepoxide; the flexibilizer for controlling the degree of hardness of the cured resin was in the form of diglycidyl ether of polypropylene (DER 736); nonenyl succinic anhydride (NSA) performed the function of hardening agent and dimethylaminoethanol (DMAE), the cure accelerator. By using different quantities of the above components, resin mixtures of differing flex and hardness can be obtained.

The various components of the Spurr's resin come in a viscous liquid form. The most convenient method assimilating a particular recipe for the resin is pippetting the required

mass of the individual components into a beaker placed on a balance, and gently mixing with a magnetic stirrer to avoid bubbles. The masses of the different components used are given in Table 4.1.

Table 4. 1: Quantities of the Spurr's resin components used to make a single batch

		Full Size
Vinylcyclohexane dioxide		10 g
Diflycide Ether of Polypropylene		6 g
Nonenyl Succinic Anhydride		26 g
Dimethyleaminoethanol		0.4 g
	Total	42.4 g

4.2.1.1.1.2 INFILTRATION AND EMBEDDING

Following dehydration, the resin was encouraged to infiltrate the specimen by placing the specimens in small vials into which 500-600 µl of a Spurr's resin: ethanol solution was added. These vials were placed in a rotary agitator for 10 hours to ensure proper infiltration. Infiltration took place over a number of days by increasing the gradient of resin to ethanol (Table 4.2).

Table 4. 2: The infiltration gradients of Spurr's resin to ethanol over time

	Ethanol: Resin Solution	
Day 1	3 Parts 100% Ethanol	1 Part Spurr's Resin
Day 2	1 Part 100% Ethanol	1 Part Spurr's Resin
Day 3	1 Part 100% Ethanol	3 Parts Spurr's Resin

While infiltration was continuing on Day 3, mixing up a new batch of Spurr's Resin was required due to the short pot life of the resin. This was placed under vacuum for at least an hour before use for infiltration to dispel any bubbles out of the mixture. A portion of resin was removed and the specimens were transferred into this 100% Spurr's Resin and set back into agitation for another 10 hours. The remaining new resin was returned to the vacuum to continue degassing overnight in preparation for embedding.

After the 100% Spurr's Resin infiltration period, the specimens were transferred from their vials to silicone moulds, which were filled with the degassed 100% Spurr's resin. The specimens were orientated accordingly per body part as occasionally specimens that are round or ring shaped in nature, without structures which could anchor into the embedding material, have been known to drop out of the material when the section is floated before transferral to slides. Once the specimens were orientated appropriately, the moulds were placed in an oven set between 60°C and 65°C where the resin was allowed to cure for 12 hours.

4.2.1.1.1.3 SECTIONING

A Laboratoire Kastler Brossel *(*LKB) Knife-Maker 7801B was used to make glass knives from glass strips 38 X 7 mm in dimesion. A boat was created by securing foil tape to the triangular blades and sealed with wax. The knives were fixed into the Ultramicrotome, and the boat was carefully filled with water using a syringe until the meniscus inverted, giving a silver sheen.

The specimen blocks were removed from their silicone moulds, trimmed accordingly for sectioning and loaded into the ultra-microtome. Unfortunately, obtaining "gold sections" proved to be virtually impossible as the sections tended to split and disintegrate on the water's surface, even before transferral to micro-grids. A block of pure Spurr's Resin was sectioned in order to determine if the quality of the Spurr's resin was sound. Ultra-thin sections from this pure resin were obtained with relative ease. This suggested that there were penetration and infiltration problems of the resin into the specimen, or uneven fixation of the specimens.

Semi-thick (blue) section attempts proved marginally more successful. These were lifted on micromeshes, and placed immediately, unstained, into a TEM, to determine if the specimen was perhaps dropping out of the sections, thus causing instability in the section. Even though the sections were unstained, the specimen was still visible (proving that the specimen had not dropped out), and further still, striations were even visible before the electron beam destroyed the specimens. However, this form of investigation proved to be very inconsistent or unreliable, and not many micrographs could be readily obtained.

4.2.1.1.2 SCANNING ELECTRON MICROSCOPY

4.2.1.1.2.1 SPECIMEN PREPARATION FOR SEM

Mosquito legs were chosen for cuticle thickness investigations with the SEM. All specimens were washed twice in 70% ethanol in order to clean them thoroughly. The legs were given a light brushing while under the ethanol to remove some of the scales that cover the legs in order to facilitate a cleaner cut. Tarsomere I on the left middle leg was perpendicularly severed at the midpoint in a drop of alcohol using a platinum-coated blade (Figs. 4.1 and 4.2). Fresh ethanol was gently passed over the section to remove any debris. The leg was left attached to the body. Each mosquito was then placed ventral side up in a foam critical point drying container with the sectioned portion of the leg orientated vertically. The containers were then passed through 70%, 80%, 90% and 100% ethanol, attempting to agitate the contents as little as possible. Each dehydration step lasted at least two hours, and was repeated twice. The final change of 100% ethanol was allowed to stand overnight, and the ethanol used was kept under a molecular sieve to ensure complete dehydration. The specimens were then sent for critical point drying (CPD).

Following CPD, the specimens were mounted ventral side up on SEM viewing stubs, taking care to ensure vertical positioning of the sectioned part of the leg to enable a

square measurement across the section from above the stage. This often required burying potions of the leg in the mounting transfer for stability reasons.

Specimens were sputter coated with carbon and gold palladium prior to viewing. Viewing was initially performed using a Jeol JSM-840, but later access to a newer SEM, the FEI Quanta 400 E scanning electron microscope, became available, allowing for higher resolution micrographs. Micrographs obtained from the Jeol JSM-840 were digitized (those from the Quanta 400E were already in digital format) using Adobe Photoshop and examination proceeded using Zeiss AxioVison Release 4.6 software to measure the thickness of the cuticle (Figure 4.3). Measurements were made by tracing the outline of both the inner and outer circumference of the cuticle and measuring the shortest distance between the two at no fewer than 25 different, evenly distributed points, with obvious aberrations such as scale beds etc. excluded. A mean cuticle thickness per specimen was obtained in this way.

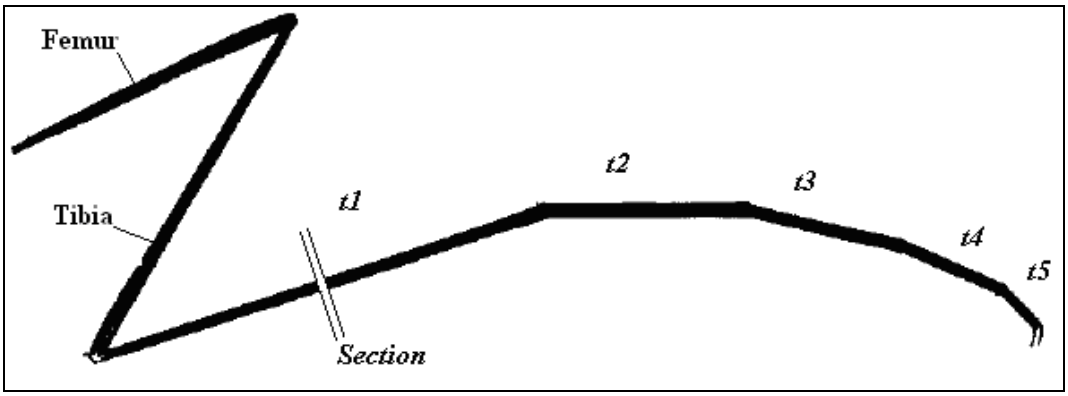

Figure 4. 1: Illustration of the point of section on *An. funestus* tarsomere 1 (*t1-t5* = five tarsal segments).

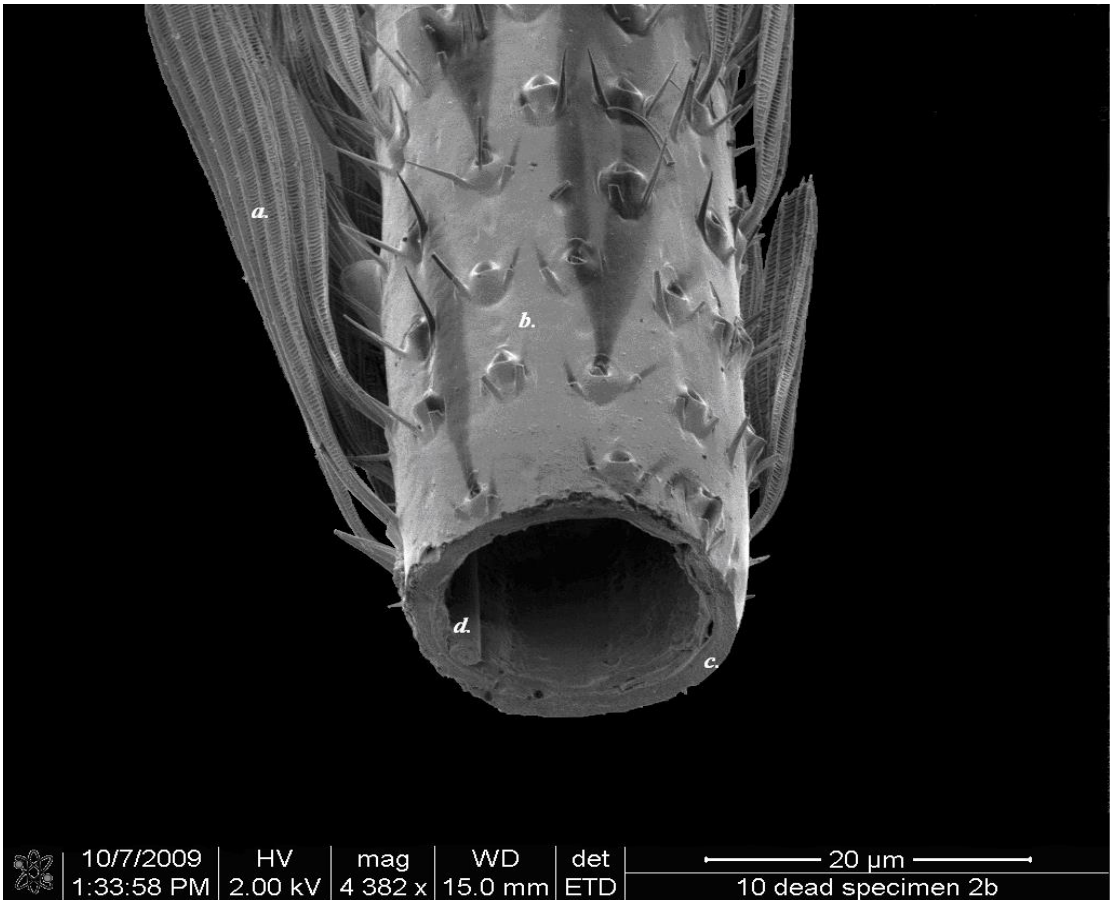

Figure 4. 2: SEM illustrating an oblique view of a sectioned first tarsal segment of the left middle leg. a) Scales which cover the leg. b) Area where scales were removed to facilitate a cleaner sectioning. c) The integument. d) Muscle or tendon (if the tarsal segment was not cut at exactly at the halfway point, this feature was either not present or did not touch the wall of the leg. As such it served as both quality control and as a reference point for standardizing points of measure).

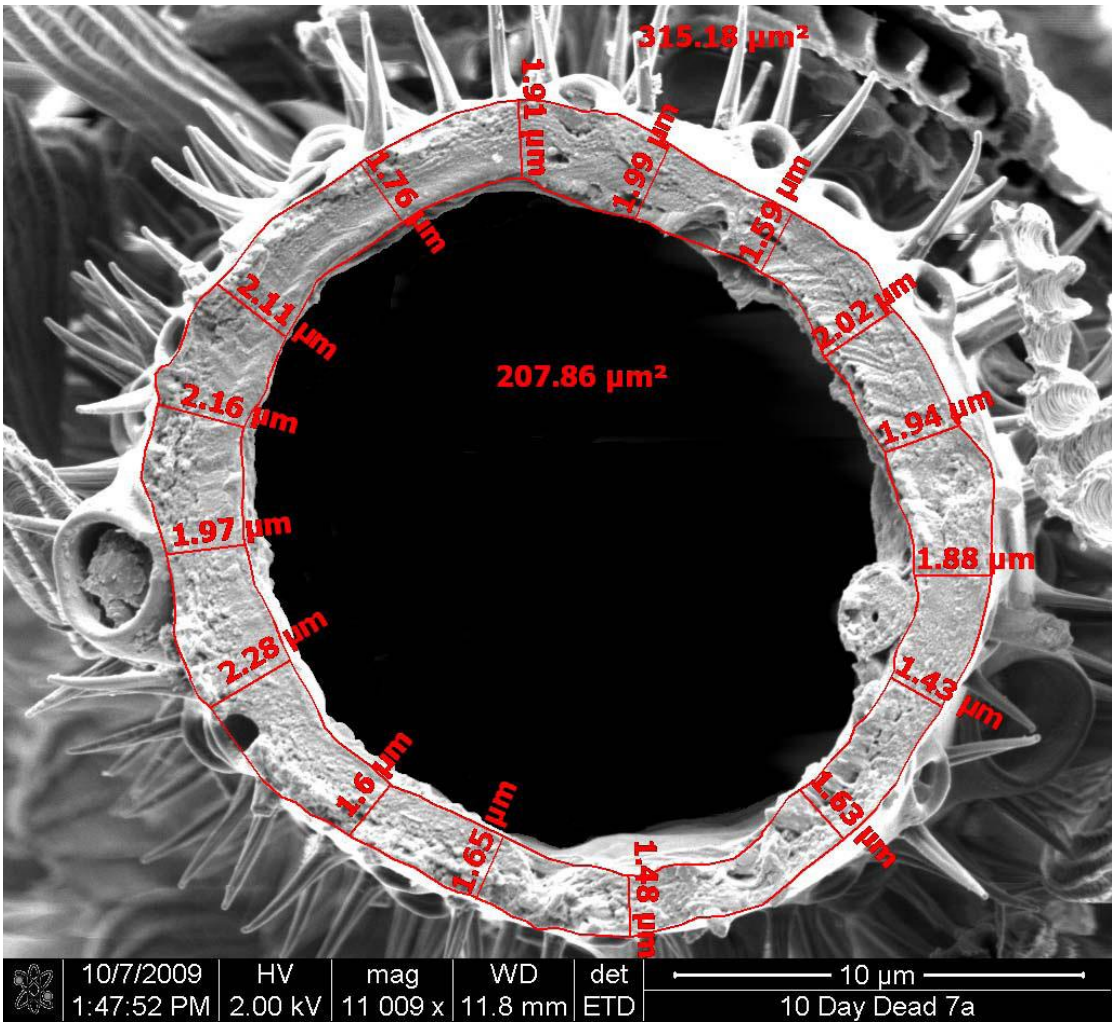

Figure 4. 3: Scanning electron micrograph from the FEI Quanta 400 E SEM of a cross-section of *An. funestus* tarsomere 1. Mean cuticle thickness was obtained from several thickness measurements (at least 25 per specimen) between the inner and outer cuticle surfaces. Area measurements include the surface area of the region identified as cuticle, which is given as 112.52 µm^2 for this specimen.

4.2.2 PRELIMINARY TRIALS: RESULTS AND CONCLUSION

Problems concerning uneven fixation and resin infiltration needed to be overcome. In addition, it was necessary to choose an appropriate body region for cuticle thickness measurements. Adult mosquitoes absorb insecticide from treated surfaces through their footpads and possibly wings. Given the complex structure of the footpads, it was decided to measure the cuticle thickness of tarsomere 1 (Fig. 4.1) based on the assumption that variation in cuticle thickness in the footpads in association with response to insecticide exposure would also be reflected in the cuticle thickness of the legs. However, the sectioning of tarsomere 1 posed a number of difficulties including orientation of the left middle leg in the resin block. Each leg for sectioning needed to be orientated at a right angle to the surface being sectioned in order to avoid the measurement bias caused by a cross section that was not exactly perpendicular. The difficulty was ensuring and maintaining the orientation of the legs after embedding and during the curing period. The mid-point of tarsomere 1 was chosen for consistency, but obtaining the midpoint of each specimen required repeated trimming of the resin block, thus presenting the same dangers or difficulties mentioned.

With SEM imaging, despite the occasional aberration, it was found that with minor tweaking of the technique and practice many of these problems could be addressed. This Made this form of imaging suitably reliable and repeatable. When balanced against the amount of time and effort required for preparation needed for light or TE microscopy, SEM proved to be the best of the options available.

4.3 PRIMARY OBJECTIVE ASSAYS

4.3.1 PERMETHRIN TOLERANCE ASSAY

Samples of *An. funestus* adults were drawn from the FUMOZ colony described earlier. In order to choose an appropriate age for cuticle thickness measurements, the findings of Cook *et al*. (2006) concerning the expression periods of cuticle gene orthologue Ae-8505 in *Aedes aegypti* were used as a guide. It was decided to use ten-day-old female mosquitoes all drawn from the same FUMOZ cohort. This age cohort was chosen in an effort to minimise variation in thickness due to age dependent gene expression as well as to obtain cuticles at their thickest considering growth and repair.

Twenty females were aspirated into a WHO exposure tube containing a 0.75% permethrin treated filter paper supplied by the World Health Organization (WHO). Knockdown of mosquitoes in the exposure tube was continuously monitored for one hour. In order to validate a knockdown of an individual, the base directly under the mosquito was lightly tapped in order to ascertain whether it was a true knockdown. Any individual that was still able to fly was kept in the exposure tube until it became completely moribund, at which point it was removed to an individual container using an aspirator carefully inserted into the tube. The time of removal was recorded. Those individuals that were still active after the full 60 minutes exposure period were grouped as +60 minutes. All samples were cold-terminated in a fridge at 4°C within five minutes of removal from the permethrin exposure tube. This was done to ensure that further

cuticle formation in any of the mosquitoes was prevented. Females representing the earliest knock-down cohort (<30 minutes to knock-down) were grouped as less tolerant, whilst females with a knock-down time in excess of 40 minutes were grouped as more tolerant. This process was repeated a number of times to increase the number of samples available from each grouping.

Specimen preparation and viewing on the Jeol JSM-840 SEM were performed as described in the previous section. The permethrin tolerant and susceptible cohorts were then compared using one-way ANOVA and a possible trend between cuticle thickness and time to knock-down was evaluated using linear regression (Statistix 7.0 software).

4.3.2 PERMETHRIN SUSCEPTIBILITY ASSAY

Following the positive results in the tolerance study, where thicker cuticles were more likely to be observed in permethrin tolerant phenotypes, a permethrin susceptibility study was conducted. After 9 days, approximately 100 FUMOZ females were exposed 25 at a time to 0.75% permethrin for one hour. The exposed females were allowed a 24 hour recovery period after which the now ten day old survivors were removed to a container and cold terminated. A similar procedure was repeated with ten-day-old females from the same population. Those ten day old females that died immediately following exposure were removed and cold stored.

These two phenotypically characterised groups were then prepared for cuticle thickness measurements and viewed on the Jeol JSM-840 as previously described. The permethrin resistant and susceptible cohorts were then compared using one-way ANOVA.

4.3.3 GENDER COMPARISON

Given the variation in insecticide resistance phenotypic expression between males and females in southern African *An. funestus* (females are generally more tolerant/resistant (Hunt *et al*., 2005), it was decided to ascertain whether there was significant variation in cuticle thickness by gender.

A sample of males and females was removed from a cohort of the FANG colony within a few hours of emergence. This colony was chosen for use in this experiment because it does not carry any measurable insecticide resistance as a confounding variable when comparing cuticle thickness between the genders. The sample was aged to five days before being cold terminated as in previous assays. The wing lengths of all the individuals used for subsequent measurements were determined rejecting those outside a significant difference of P=0.07. This was done to account for or eliminate body size bias on cuticle thickness (Lyimo & Takken, 1993).

All specimens were prepared for SEM as described earlier, and the FEI Quanta 400 E scanning electron microscope was used to obtain digital micrographs. The images on all micrographs were analysed and measured as described earlier and the mean cuticle

thickness of each specimen was calculated. Mean cuticle thickness was compared between males and females using one-way ANOVA (Statistix 7.0 software).

4.3.4 CUTICLE TO AGE RELATION

As insect cuticle is laid down in a circadian fashion, and that it is often observed that older individuals exhibit greater resistance to insecticides up to a certain age, it seemed plausible that older females may have thicker cuticles too.

A sample of 4th instar FANG larvae were removed and reared to adults. The females were removed within two hours of emergence and placed at even densities in three cages, where they had access to a 10% sugar solution in a cotton wool pads. The females in the respective cages were then killed in the refrigerator at 3, 5, and 10 days of age respectively.

Preparation for viewing the cuticle thickness proceeded as previously outlined. Unfortunately, at the time of the experiment an accident by another user of the FEI Quanta SEM required it to be down for maintenance for an indefinite period. This required returning to the JOEL, which was in a state of being decommissioned as the Quanta was due to replace it. Unfortunately, owing to excessive charging up of the legs, it was particularly difficult to obtain clear and appropriately balanced micrographs. As a result, and in addition to this, frequently, it was not possible to obtain micrographs at an workable magnification.

Due to the flooding, and the static, grainy quality, and low magnification of the micrographs, after converting the negative celluloid micrographs to digital images for analysis, obtaining and accurately outlining the inner and outer circumferences of the cuticle became very subjective and difficult. Complicating matters further, the Quanta was brought back online in time to view the legs belonging to the 5 day old females. Day three and 10 had already been examined under the JOEL.

When it came to examination of the data, it became obvious that the results were most likely erroneous, owing to the difficulties outlined above, and were not worth committing to paper. It was decided, then, to commit the question of cuticle thickness and its relation to age to future investigations.

4.3.5 RESULTS

4.3.5.1 PERMETHRIN TOLERANCE ASSAY

During viewing, it was noted that about half of the prepared specimens had internal tissue protruding beyond the sectioned edge (Fig 4.4). This had the effect of obscuring the parts of the section. As a result, measurements of nine individuals from each of the pyrethroid susceptible and tolerant samples were obtained.

The mean cuticle thickness of the permethrin susceptible group was 2.13 μm (SD ±0.10 μm) while the permethrin tolerant group showed a mean thickness of 2.33 μm (SD ±0.22 μm), giving a mean difference of 0.20 μm (Fig. 4.5). This difference is significant based

on one-way ANOVA (P=0.03). A linear regression of time to knock-down (kd_t) vs. mean cuticle thickness is shown in Figure 4.6. There is a significant trend (P=0.01) in which cuticle thickness generally increases with increasing length of time to knock-down, although the correlation is weak (R^2=0.33).

4.3.5.2 PERMETHRIN SUSCEPTIBILITY ASSAY

The mean cuticle thickness of the susceptible specimens was 2.00 µm (SD ±0.20 µm) while the resistant specimens showed a mean thickness of 2.21 µm (SD ±0.15 µm) (Figures 4.7 and 4.8). This results in a mean difference of 0.21 µm that was significant (P=0.02) based on one-way ANOVA.

4.3.5.3 GENDER COMPARISON

There was no significant variation in wing length between samples (mean wing-length of males = 3.01 mm; mean wing-length of females = 3.12 mm; P= 0.07 based on a 2 sample t test).

Mean cuticle thicknesses were 1.79 µm (SD ± 0.81 µm) for the male sample and 2.01 µm (SD ± 0.15 µm) for the female sample (Figure 4.9). The mean difference of 0.22 µm was significant based on ANOVA (P = 0.01) (Figure 4.9).

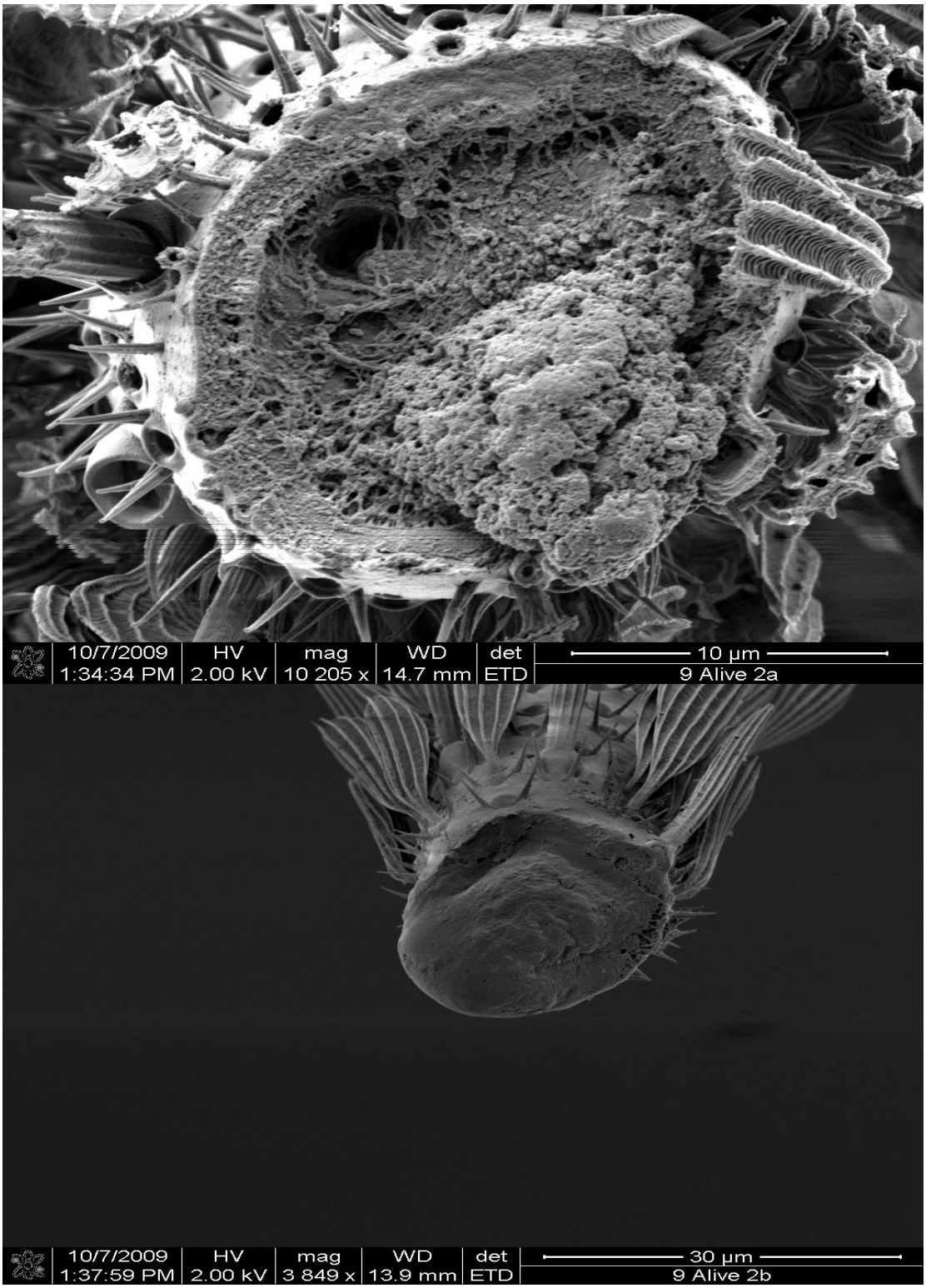

Figure 4. 4: SEM micrographs of Resistant Specimen 2 demonstrating obscuring by inner tissue on both sides of the section on the same specimen taken using the FEI Quanta 400 E

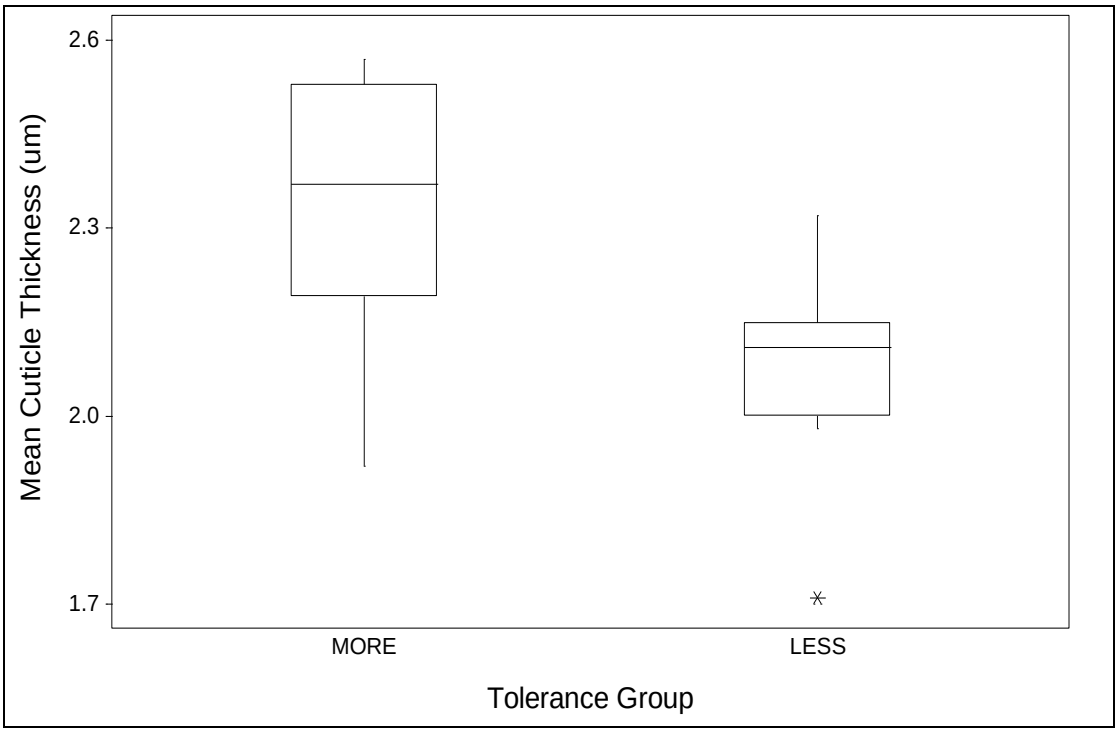

Figure 4. 5: Mean cuticle thicknesses of two samples of *An. funestus* laboratory reared females characterised as either more or less tolerant to permethrin intoxication.

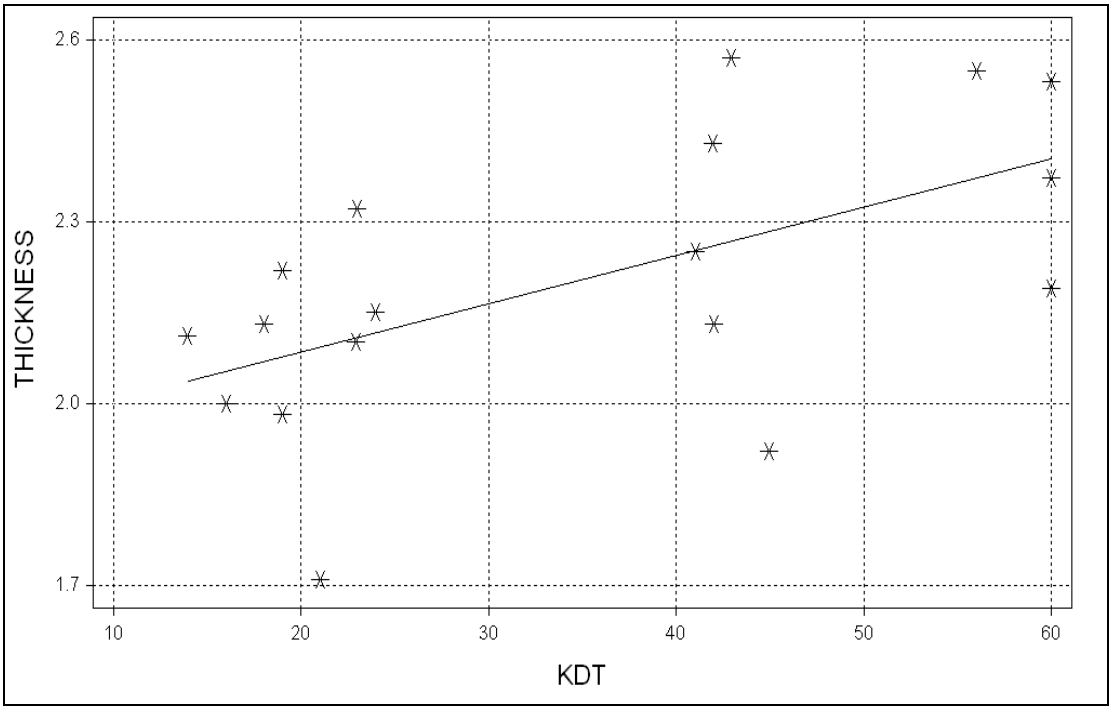

Figure 4. 6: Scatter graph of time-to-knockdown (KDT) during exposure to permethrin vs. mean cuticle thickness (microns). The trend is significant but weakly correlated (R^2=0.33, P= 0.03).

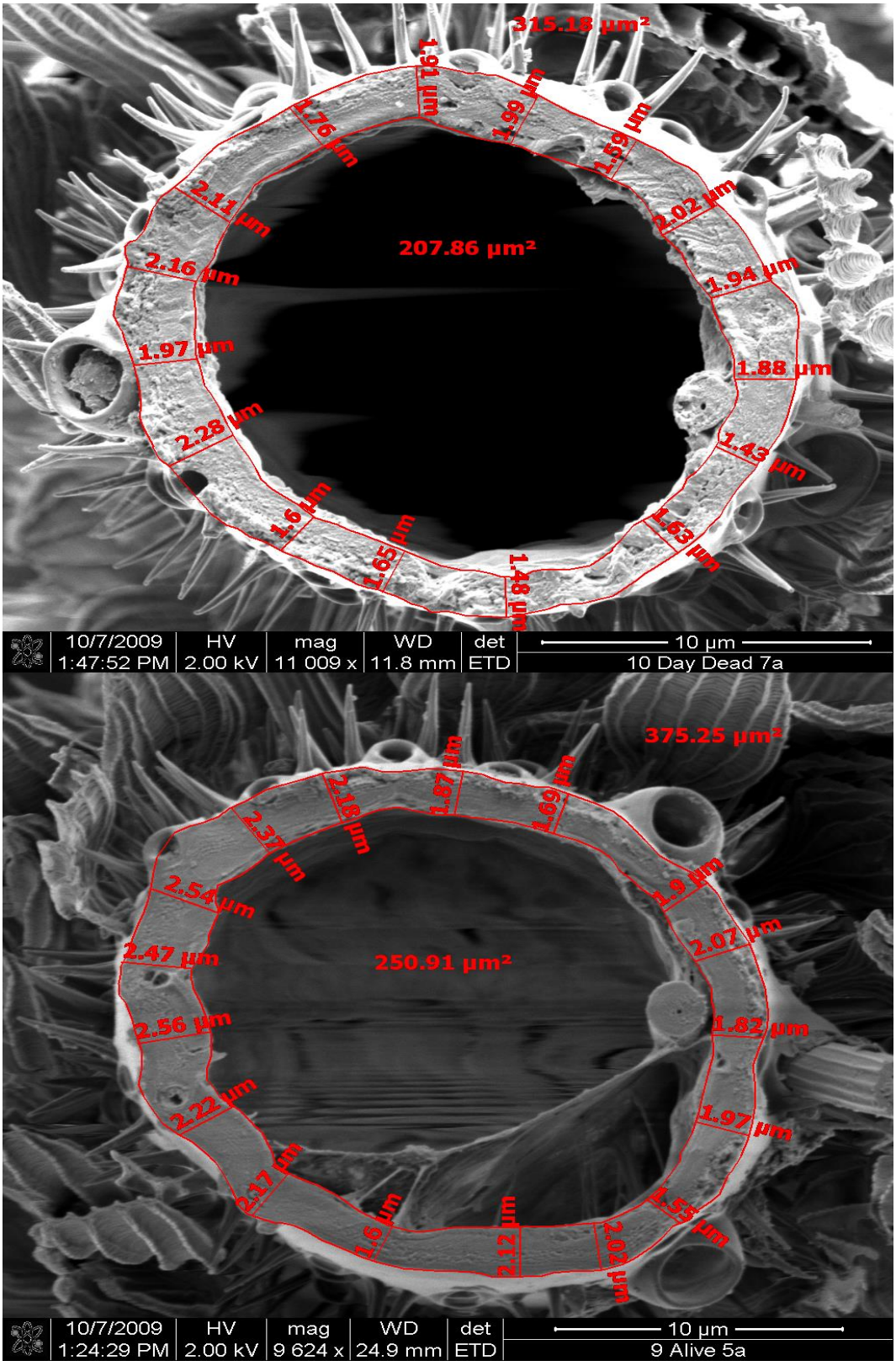

Figure 4. 7: SEM micrographs from the FEI Quanta 400 E, demonstrating the difference in mean cuticle thickness between susceptible and resistant individuals.

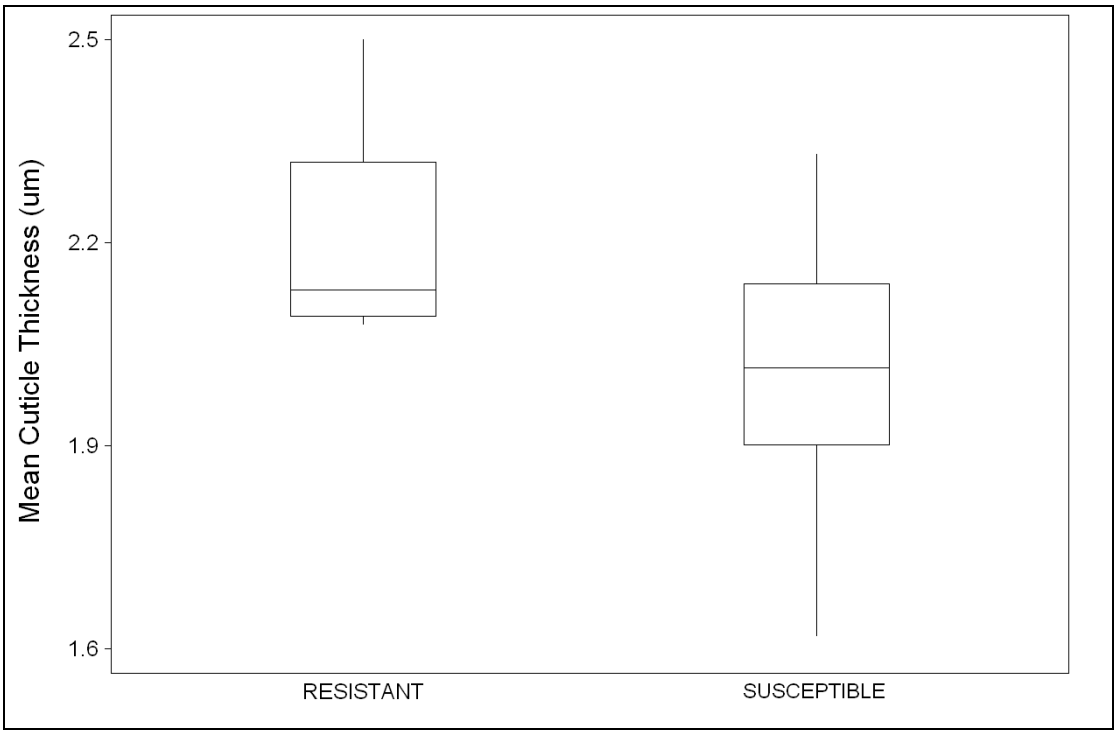

Figure 4. 8: Mean cuticle thicknesses of two samples of *An. funestus* laboratory reared females characterised as either resistant or susceptible to permethrin intoxication.

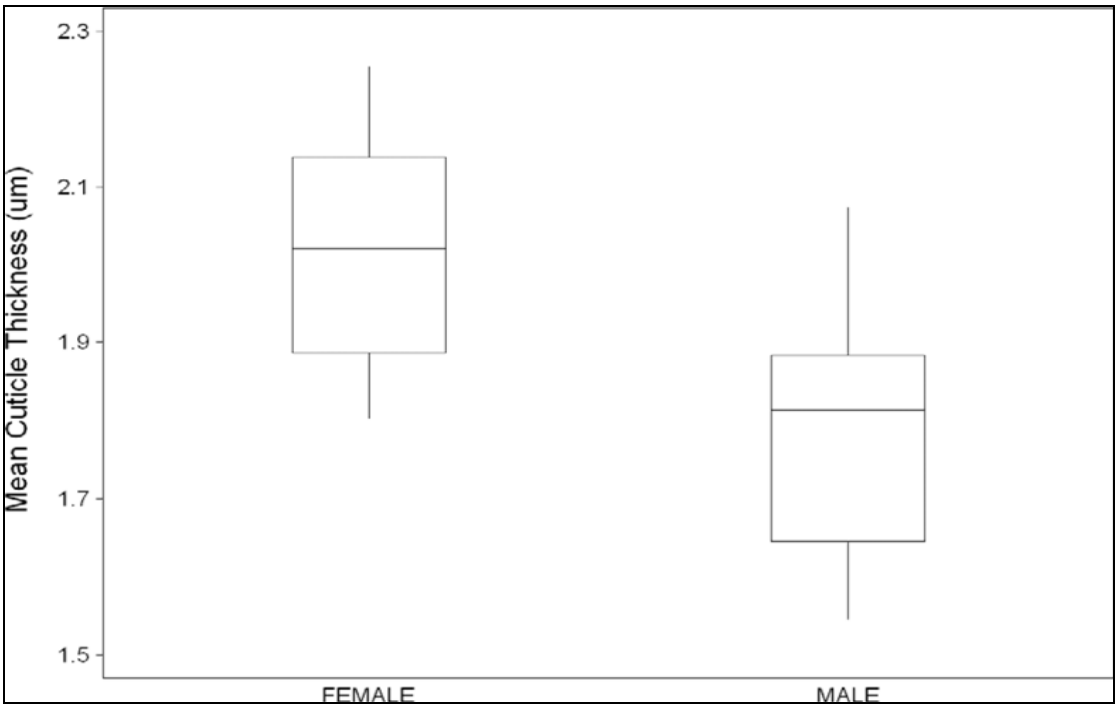

Figure 4. 9: Box and whisker plot illustrating the difference in mean cuticular thicknesses of the two genders

4.3.6 DISCUSSION

Insecticide resistant phenotypes and their underlying mechanisms tend to evolve rapidly under intense insecticide selection pressure, leading to the prediction that resistance, in general, is likely to be conferred by a small number of major effect genes (Ffrench-Constant *et al*., 2004). However, differential expression of many genes not normally associated with insecticide resistance may also occur (Vontas *et al*., 2005).

Measurements of response to permethrin exposure, expressed either in terms of tolerance during exposure or outcome following exposure, show similar associations with mean cuticle thickness. There appears to be a degree of conservation in the difference, ~0.2 µm, in cuticle thickness between phenotypes. There was a 9.5-10% increase in mean cuticle thickness in those individuals either more tolerant or resistant to permethrin compared to those that were either less tolerant or permethrin susceptible. A link between cuticle thickness and response to insecticide exposure is thus based on the assortment of phenotypes, whereby the permethrin tolerant and resistant groups were significantly associated with thicker cuticles and vice versa. A similar difference in cuticle thickness was observed between insecticide susceptible males and females, which may contribute to differences in tolerance to insecticide intoxication generally observed between genders in *An. funestus* (Hunt *et al*., 2005). Admittedly in certain cases the sample size for individual studies turned out to be limited as a result of specimen viewing quality, but as the collection of the individual studies corroborate each other, this position can possibly be considered to be strengthened. Variation in the expression of pyrethroid resistance

with age in the FUMOZ colony is most likely based on variation in monooxygenase gene expression. Ten-day-old FUMOZ mosquitoes show reduced insecticide resistance compared to younger cohorts (Hunt *et al*., 2005), but these data give no indication as to whether this variation is in any way linked to cuticle deposition.

These data support the hypothesis that the efficiency of monooxygenase-based pyrethroid resistance in southern African *An. funestus* is likely to be affected by minor factors. This is because in order to produce an effective resistant phenotype, upregulated transcription of selected P450 monooxygenase genes must produce sufficient enzyme to catalyze the metabolism of pyrethroids at a rate that prevents significant interaction between the insecticide and its neuronal target. Therefore, any mechanism that slows or regulates insecticide inoculation of internal organs, such as cuticular thickening, is a likely candidate for selection along with the primary mode of resistance.

The effect of such a reduction in penetration of insecticide has many implications when it comes to vector control. Other than insecticide treated bed-nets, indoor residual spraying (IRS) is a primary control method. When engaging in IRS, fast acting insecticides that affect a quick knockdown of the mosquitoes alighting on the wall within the structure are favourable. These are favoured both for efficacy in killing the mosquito but may also reduce selection for resistance to insecticides through sub-lethal doses. The issue of sub-lethal doses becomes particularly important when considering possible retardation and hence uptake of insecticide caused by thickened cuticle (Ahmed *et al*., 2006, Wood *et al*., 2010).

4.4 CONCLUSIONS

It can be concluded that pyrethroid tolerant or resistant *An. funestus* females are likely to have thicker cuticles than less tolerant or susceptible females, and that females generally have thicker cuticles than males. In pyrethroid resistant *An. funestus*, this increase in cuticle thickness is likely to have developed as an auxiliary to the primary mode of resistance, which is based on enzyme-mediated detoxification.

This knowledge may better aid in the design of effective resistance management strategies in the future, which aim to either circumvent or reduce the rate of insecticide resistance development in vector populations, with a more complete understanding or the resistance mechanisms and their potential effects.

CHAPTER 5- GENERAL DISCUSSION

5.1 GENERAL DISCUSSION

It has already been established that resistance to pyrethroid insecticides in a population of *Anopheles funestus* in southern Mozambique enabled a range expansion of this species into northern KwaZulu-Natal after the use of DDT for Indoor Residual Spraying (IRS) programmes in South Africa was suspended and substituted with pyrethroids (Hargreaves *et al.*, 2000; Brooke *et al.*, 2001; Coetzee *et al.*, 2013). This however was already an advanced step in the lead-up to South Africa's 1996-2000 malaria epidemic. What happened earlier in this progression was the selection for pyrethroid resistance in *An. funestus* in southern Mozambique, raising the question as to how and at what life stage resistance selection occurred.

For historical reasons, largely relating to the effects of the Mozambique civil war, no formal insecticide based malaria vector control programmes were operational in Mozambique during or before South Africa's 1996-2000 epidemic. There are therefore two possible sources of selection. The first is the informal use of insecticides for malaria control purposes (e.g. mosquito coils) which would have been directed against adult mosquitoes. The second is the leaching of insecticide into *An. funestus* breeding sites as a consequence of agricultural use of insecticides, leading to selection for resistance at the larval stage.

In order to test the likelihood of these two selection pressure candidates, experiments were designed to establish whether resistance selected at the larval stage would translate into resistance in adults and vice versa. Experiments were also designed to establish if any cuticle thickening in adults also contributed to expression of the resistance phenotype at the adult stage.

5.1.1 RESISTANCE PHENOTYPE SELECTION BY LIFE STAGE

In these investigations, a combination of CDC bottle assays and WHO larval insecticide resistance monitoring techniques were employed. The baseline levels of resistance for three strains of *An. funestus* were established using the LC or LD 50s as the point of comparison. The strains used were the fully insecticide susceptible FANG originating from Angola, the partially pyrethroid-resistant FUMOZ strain originating from southern Mozambique, and FUMOZ-R which was selected for high levels or pyrethroid resistance from FUMOZ. By comparing the baseline LD50s for the adults, the expected significant variation in resistance levels was observed, i.e. FANG showed the lowest LD50 followed by FUMOZ followed by FUMOZ-R. The larvae from the three strains, however, showed no significant variation in LC50. This suggests that the characteristics and mechanisms allowing for the expression of the resistance phenotype are only expressed in the adult life stage.

Using the established LC or LD50s for the various *An. funestus* strains and life stages, samples from each life stage were exposed to permethrin at their relevant LC or LD50

levels in order to observe if selection at one life stage conferred an increase in resistance or tolerance at the following life stage. The results from these experiments were less clear than those observed in the baseline comparison.

The data showed that a single permethrin exposure did not increase resistance/tolerance in the alternate life stage in any of the *An. funestus* colonies. This may have occurred because FANG does not carry selectable permethrin resistance alleles whilst permethrin resistance in FUMOZ-R is likely fixed, precluding the possibility of enhancing resistance by selection in the adults of this colony any further. Alternatively, adults accruing from permethrin exposed larvae may have been less tolerant to insecticide because of adverse physiological effects caused by permethrin intoxication at the larval stage. This may explain the measurable decrease in permethrin resistance in FUMOZ adults following selection in adults of the previous generation.

Nevertheless, it is clear that the permethrin resistant phenotype in FUMOZ and FUMOZ-R is only expressed during the adult life stage. This would indicate that the mechanisms conferring resistance only become active in the adult life stage, suggesting that the selection pressures which lead to the development of resistance in adults must have been active against adults and not larvae, although it should be noted that these evaluations were performed on laboratory *An. funestus* strains which may not contain the same degree of genotypic and phenotypic variation as the wild populations from which they were derived. Possible sources of selection against adults are commercially available pyrethroid based mosquito coils and aerosols.

5.1.2 CUTICLE THICKENING IN ADULT *ANOPHELES FUNESTUS*

Cuticle thickening in adult mosquitoes is a somewhat neglected mode of insecticide resistance or increased tolerance to be examined. In addition to the already well-described primary mechanisms conferring pyrethroid resistance in adult FUMOZ and FUMOZ-R, it was hypothesised that thickened cuticles may also play a role in the expression of pyrethroid resistance in adults.

As little work had previously been conducted in this field, a variety of approaches were initially pursued and evaluated. A reasonably repeatable and robust technique was developed allowing for visualization of a section of cuticle most likely to be representative of portions that would come into contact with insecticide residues, using a scanning electron microscope.

The results of experiments devised to assess an association between thickened cuticle and increased tolerance or resistance to pyrethroids showed that resistant adults are more likely to have thickened cuticles than their susceptible counterparts. The correlation was found to be comparatively weak, suggesting that this mechanism is not a primary resistance mechanism but rather an auxiliary to the primary mode of pyrethroid resistance which is based on enzyme-mediated detoxification in adults.

5.2 CONCLUDING REMARKS

The mapping tool IR Mapper (www.irmapper.com) very clearly illustrates how many instances of insecticide resistance have been reported in malaria vector populations over time. As a consequence, malaria vector control is almost synonymous with managing insecticide resistance. This is best achieved when detailed information concerning resistance mechanisms, frequencies and geographical spread is available.

The information presented in this thesis concerning the characteristics and phenotypic expression of pyrethroid resistance in southern Africa *An. funestus* forms part of a greater body of work dedicated to understanding the bionomics of resistance in this species. Insecticide resistance in southern African *An. funestus* has now been thoroughly characterised, and has enabled ongoing and effective control of this species within South Africa despite the occurrence of resistance. Nevertheless, malaria vector mosquito populations are dynamic in terms of their adaptive characteristics, making ongoing entomological surveillance in malaria affected areas critical for the maintenance of effective control. In addition, South Africa is scheduled to achieve malaria elimination within its borders by 2018. Thus malaria vector surveillance and control measures will need to be enhanced, as will operational research activities designed to better understand those adaptive characteristics in malaria vector populations that are of epidemiological significance, especially insecticide resistance.

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APPENDIX A: PUBLICATION LINKED TO CHAPTERS 2 AND 3

Contribution to publication:

Oliver Wood performed and analyzed the results of WHO insecticide bioassays on FANG and FUMOZ-R 4th instar larvae. These data in conjunction with the data of CDC bottle bioassays for the corresponding adults (supplied by Belinda Spillings) and the molecular evidence were all supporting that the resistance phenotype is expressed during the adult life stage but not the larvae.

Insect Molecular Biology (2008) **17**(1), 19–25

Over expression of a Cytochrome P450 (CYP6P9) in a Major African Malaria Vector, *Anopheles Funestus*, Resistant to Pyrethroids

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Abstract

Anopheles funestus **Giles is one of the major African malaria vectors. It has previously been implicated in a major outbreak of malaria in KwaZulu/Natal, South Africa, during the period 1996 to 2000. The re-emergence of this vector was associated with monooxygenase-based resistance to pyrethroid insecticides. We have identified a gene from the monooxygenase CYP6 family, CYP6P9, which is over expressed in a pyrethroid resistant strain originating from Mozambique. Quantitative Real-Time PCR shows that this gene is highly over expressed in the egg and adult stages of the resistant strain relative to the susceptible strain but the larval stages showed almost no difference in expression between strains. This gene is genetically linked to a major locus associated with pyrethroid resistance in this** ***A. funestus*** **population.**

Keywords: P450, insecticide resistance, mosquito.

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Introduction

Malaria remains one of the devastating diseases in Africa and malaria control programmes rely extensively on insecticide-based vector control. Insecticides are mainly employed in indoor residual spraying (IRS) or applied to bednets (insecticide treated nets/ITNs). The importance of indoor residual spraying to control vector mosquitoes was reiterated recently by W.H.O (www.who.int/mediacentre/news/releases/2006/pr50/en/). Pyrethroid insecticides remain the first choice of chemical for IRS due to their low mammalian toxicity, rapid breakdown in the environment and efficacy and are also the only class of chemicals allowed for use on impregnated bednets. However, pyrethroid resistance in vector mosquitoes has recently been reported throughout Africa including South Africa (Hargreaves *et al.*, 2000), Nigeria (Awolola *et al.*, 2002), Cameroon (Chouaibou *et al.*, 2006), Cote d'Ivoire (Tia *et al.*, 2006), Ghana (Coetzee *et al.*, 2006) and Benin (N'Guessan *et al.*, 2007).

Because of the rapid and widespread development of resistance to insecticides in target vector populations, insecticide resistance management is becoming an integral part of vector control. This strategy is designed to monitor and circumvent the development of insecticide resistance in affected populations as well as to prevent the development of resistance in unaffected populations. In the absence of resistance management, the impact of insecticide resistance on malaria transmission can be severe. Such an impact was clearly demonstrated in KwaZulu/Natal, South Africa, when pyrethroid resistant *Anopheles funestus* Giles re-emerged during the late 1990's leading to a severe malaria epidemic during the period 1996 to 2000. This occurred despite ongoing control efforts using pyrethroid insecticides (Hargreaves *et al.*, 2000; Coetzee & Fontenille, 2004). *A. funestus* is the nominal member of the funestus group and, along with *Anopheles arabiensis* Patton, is a major vector of malaria in the southern African region. *A. funestus* is highly anthropophilic and endophilic making it especially vulnerable to control by IRS assuming effectiveness of the insecticide employed (Gillies & DeMeillon, 1968).

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Pyrethroid resistance can arise due to mutations in the target site, the sodium ion channel gene, or up-regulation of metabolic enzymes, mainly monooxygenases which detoxify the insecticide. There are no reports of target site resistance in *A. funestus* to date. Detoxification of pyrethroids in insects is mainly accomplished by monooxygenases, also called P450 enzymes. Over expression of one or more of these P450 genes has been associated with pyrethroid resistance in house flies (Kasai & Scott, 2000), mosquitoes (Nikou *et al.*, 2003; David *et al.*, 2005) and many other insects. Majority of these genes are from four main families, CYP4, 6 and 9 (Feyerseisen *et al.*, 1989). During the last 18 years since the isolation of the first insect P450 in the housefly *Musca domestica* (Feyereisen *et al.*, 1989), over 300 P450s have been identified in insects (http://p450.antibes.inra.fr/). Insect P450s are distributed across 48 CYP families which include CYP4, CYP6, CYP9, CYP12, CYP18, CYP28, CYP49 and CYP301-341 (http://drnelson.utmem.edu/CytochromeP450.html). Identification of this P450 diversity in insects was accelerated by the genomic sequencing of *Drosophila melanogaster* and *A. gambiae* (Tijet *et al.*, 2001; Ranson *et al.*, 2002). In *A. funestus*, 31 partial P450 genes were isolated using degenerate primers based on *A. gambiae* P450 sequences (Amenya *et al.*, 2005).

Monooxygenase-based resistance to pyrethroids in southern African *A. funestus* was first demonstrated by Brooke *et al.* (2001). The controlling resistance factor appears to be inherited as a single locus, autosomal, incompletely dominant phenotype (Okoye *et al.*, unpublished data). Quantitative trait locus (QTL) mapping of the phenotype identified a major pyrethroid resistance locus on chromosome arm 2R, coinciding with the location of a cluster of CYP6 genes mapped by *in situ* hybridisation (Wondji *et al.*, 2007). These data support the hypothesis that pyrethroid resistance in southern African *A. funestus* is mediated by one or a cluster of P450 enzymes specifically from the CYP6 family. In the current study, we used hybridisation and PCR techniques to identify a specific P450 gene that is over expressed in the pyrethroid resistant strain.

Results

In initial experiments, serial dilutions of RNA from the resistant and susceptible strains were hybridised with cocktail probes from the CYP4, 6 or 9 classes. These four classes were targeted based on their association with pyrethroid resistance in other insect species such as *A. gambiae*, *M. domestica* etc. (Feyerseisen *et al.*, 1989). The cocktail probes consist of those subfamilies isolated and sequenced from cDNA by Amenya *et al.* (2005) as this paper is an extension of the same project. A detectable signal was only observed with the CYP6 cocktail probe and only in the resistant strain (Fig. 1). Expression of CYP6 P450s was

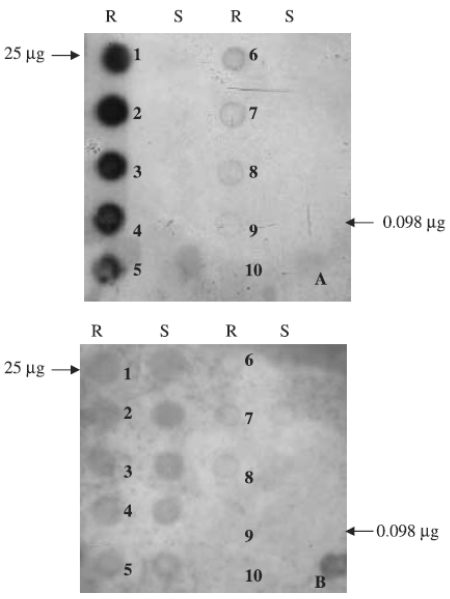

Figure 1. Dot blot analysis of 3-day old adult FUMOZ-R (R) and FANG (S) RNAs. Autoradiogram after 5 h exposure. (A) Autoradiogram showing hybridisation results with cocktail CYP6 probes. (B) Autoradiogram showing hybridisation result with rsp7 probe. RNA concentrations (1 = 25 µg, 2 = 12.5 µg, 3 = 6.25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

clearly visible with 1.5 µg of the total RNA from the FUMOZ-R strain whereas no signal was observed even at the highest concentration (25 µg) in the susceptible strain. The CYP6 primer cocktail was prepared from the partial sequences of six CYP6 genes from three different subfamilies (CYP6P, CYP6Z and CYP6M). We subsequently prepared single gene probes and used these to compare gene expression between the FUMOZ-R and FANG strains. The starting RNA concentration was increased to 50 µg. When using a probe for the CYP6P9 gene, expression was clearly visible even at low concentrations of FUMOZ-R RNA while remaining almost undetectable in the FANG strain (Fig. 2). No signal was detectable in either strain when using single probes from the remaining five CYP6 genes (data not shown). Over expression of the CYP6P9 gene was confirmed using northern blot analysis (Fig. 3) which showed a single transcript of approximately 2 kb with expression visibly higher in the FUMOZ-R strain.

To quantify the difference in expression of CYP6P9 between the two strains primers were designed to amplify

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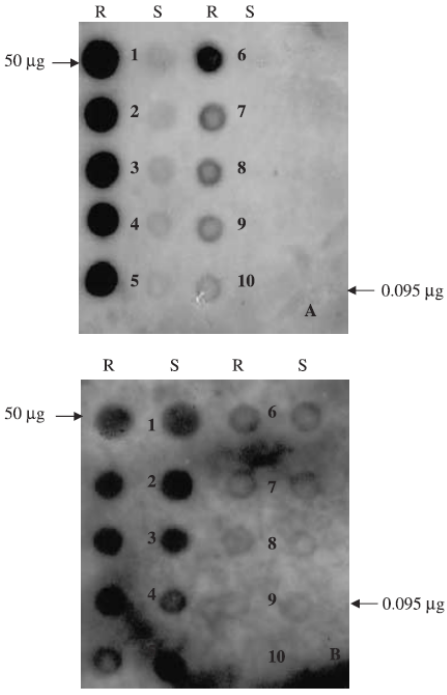

Figure 2. Dot blot analysis of 3-day old FUMOZ-R (R) and FANG (S) RNAs. Autoradiogram after overnight exposure. (A) Autoradiogram showing hybridisation results with CYP6P9 probe. (B) Autoradiogram showing hybridisation result with rsp7 probe. RNA concentrations (1 = 50 µg, 2 = 25 µg, 3 = 12.5 µg, 4 = 6.25 µg, 5 = 3.125 µg, 6 = 1.56 µg, 7 = 0.78 µg, 8 = 0.39 µg, 9 = 0.195 µg, 10 = 0.098 µg).

a 140 bp fragment from the CYP6P9 gene and 135 bp for rsp7 and used in real time PCR reactions using cDNA from both strains as a template. Melting curve analysis showed a single product specific melting temperature of 87.5 °C and 89 °C for CYP6P9 and rsp7 respectively. No primer dimers were observed. The products were sequenced to confirm that the expected products have been amplified.

Figure 4 shows the results of the quantitative PCR analysis. CYP6P9 expression was elevated in the resistant FUMOZ-R strain compared to the susceptible FANG strain in the egg and adult stages. However, expression of this gene was approximately equal in the two strains in all larval stages and in the pupal stage (Fig. 4). Elevated expression was particularly distinct in the eggs and 14 day old adults. When comparing the sexes, expression of CYP6P9 was higher in females than males.

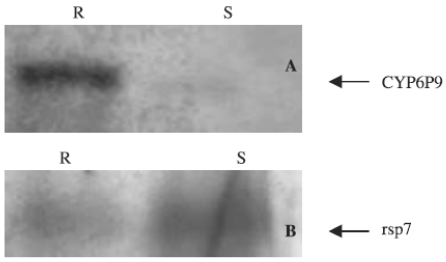

Figure 3. Northern blot analysis of CYP6P9 mRNA levels in FUMOZ-R (R) and FANG (S) strains. RNA (5 µg) was loaded in each lane (A) CYP6P9 probe (B) rsp7 probe.

The quantitative PCR analysis was also performed using genomic DNA as a template. The ratio of expression between CYP6P9 and rsp7 was approximately 1:1 in both mosquito populations (data not shown) suggesting that the increased production of mRNA in the FUMOZ-R strain is not due to gene duplication.

WHO bioassay exposures using 0.75% permethrin against three day old FUMOZ-R adults showed similar mortality rates between females (22%) and males (28%). However, 14 day old adult mortality was 86% in females and 94% in males. An insecticide dose response comparison between 3–5 day old FUMOZ-R and FANG adult females gave permethrin LD_{50}-values of 141.2 µg/250 ml glass bottle (SE = 17.9) for FUMOZ-R and 1.2 µg/250 ml glass bottle (SE = 0.3) for FANG. These data represent a 118 fold difference in adult response ($P < 0.05$) between the two strains.

Bioassays comparing FUMOZ-R and FANG 4th instar larvae gave permethrin LC_{50}-values of 10.8 µg/ml (SE = 0.17) for FUMOZ-R and 4.3 µg/ml (SE = 0.07) for FANG. This represents only a 2.5 fold difference in response ($P = 0.01$) between the two strains.

Discussion

Investigating metabolic resistance mechanisms in insects is complex owing to the extensive size of the gene families involved and their rapid diversification. Amenya *et al.* (2005) isolated the first 31 partial P450 gene fragments from *A. funestus* in order to study pyrethroid resistance in this species. We have used these partial gene fragments to identify potential genes over expressed in the pyrethroid resistant strain, FUMOZ-R.

Twelve partial CYP4 genes and five partial CYP9 genes did not show any over expression in FUMOZ-R compared to FANG. This indicates that these genes do not associate with the resistance phenotype and are probably not involved in detoxification of pyrethroids in the FUMOZ-R strain.

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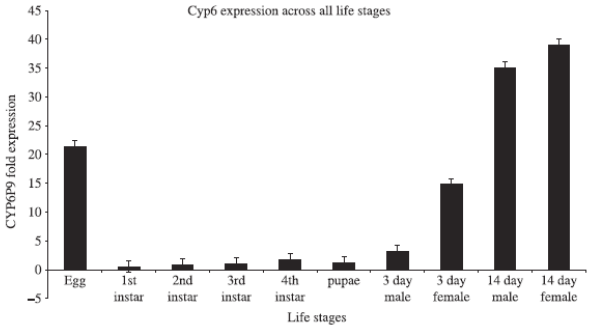

Figure 4. Quantitative PCR analysis CYP6P9 expression in *A. funestus* from the FUMOZ-R (pyrethroid resistant) and FANG (susceptible) strains. For each strain expression was normalised against rsp7 and the ratio of the normalised CYP6P9 expression is plotted. Significant differences in expression between the strains were observed in eggs ($t = 6.31$, $P < 0.05$); 4th: fourth instar larvae ($t = 8.61$, $P < 0.05$); 3 day old males ($t = 22.76$, $P < 0.05$); 3 day old females ($t = 4.72$, $P > 0.05$); 14-day old males ($t = 23.16$, $P < 0.05$) and 14 day old females ($t = 7.46$, $P < 0.05$).

Genes in the monooxygenase CYP6 family have been implicated in insecticide resistance in other insects including *M. domestica* (Feyereisen *et al.*, 1995; Liu & Scott, 1998; Kasai & Scott, 2000), *A. gambiae* (Nikou *et al.*, 2003), *Anopheles albimanus* (Rongnoparut *et al.*, 2003) and *Anopheles minimus* (Rodpradit *et al.*, 2005). Identification of the actual gene that was over-expressed in the pyrethroid resistant strain of *A. funestus* was achieved by probing each of the six genes comprising the CYP6 cocktail (CYP6P1, CYP6P2, CYP6P9, CYP6Z1, CYP6M8 and CYP6M7) separately. Only one of these, CYP6P9, was expressed at elevated levels in the resistant strain. While it is recognised that the transcription profile of only a small subset of the CYP6 family has been examined so far (*A. gambiae* contains 30 CYP6 genes) the very high level of over expression of CYP6P9 in the resistant strain (up to 38-fold in 14 day females) and the genomic location of this gene (see below) suggests that expression of this P450, which is an ortholog of CYP6P3 in *A. gambiae* (Amenya *et al.*, 2005), is associated with resistance to permethrin in *A. funestus*.

The over expression of CYP6P9 is confined to the eggs and adult stages. There was no significant over-expression in the larvae. Hence if enhanced detoxification of pyrethroids by CYP6P9 is the major causative factor of pyrethroid resistance in the FUMOZ-R strain, the resistance phenotype would be expected to be manifested in the adult stage only. Stage specificity of the resistance phenotype is supported by bioassays against larvae in which there are low levels of variation at permethrin LC_{50} concentrations between the FUMOZ-R and FANG strains. On the other hand, dose-dependent permethrin bioassays against adult females showed an enormous difference in response between the resistant and susceptible strains. Furthermore, the FUMOZ-R colony was under selection at the adult stage only which may account for the development of an adult specific protection mechanism. If resistance is only present in adult mosquitoes in field populations this may suggest that elevated expression is being selected for by use of insecticides in malaria control as opposed to contamination of breeding sites by agricultural insecticides. The expression of CYP6P9 in adult mosquitoes is dramatically increased between the ages of three days old and 14 days old and the effect is most pronounced in females. This is in contrast to bioassay results published by Hunt *et al.* (2005) where mortality following permethrin exposure was significantly higher in 14-day old adults. The biological material used for this study was selected through an additional 25 generations since the original characterisation was carried out and the current bioassay analysis supports the data of Hunt *et al.* (2005). The complexities of age-dependent differentials in enzyme expression may account for variation in the expression of the resistance phenotype with age. Mapping of permethrin resistance QTL's by Wondji *et al.* (2007) shows that the chromosomal position of CYP6P9 associates with permethrin resistance and accounts for the bulk (> 60%) of the resistance phenotype but also implies that other factors play a minor role as well.

Nikou *et al.* (2003) demonstrated that within the CYP6Z subfamily, various genes (CYP6Z1, CYP6Z2 and CYP6Z3) were found to be differentially expressed in the life stages of a pyrethroid resistant strain of *A. gambiae* from Kenya. These genes share a high identity at the amino acid level indicating a recent duplication event and may play a role in the detoxification of insecticide at these specific life stages. In adult *A. gambiae*, a CYP6Z1 gene was found to be over-expressed in the pyrethroid resistant strain.

We are currently investigating the role of CYP6P9 in the detoxification of pyrethroids in order to clarify the role of this enzyme in resistance. Given that other genes may also play a role in this complex resistance mechanism, studies are ongoing using microarrays to identify additional genes involved. The report by Wondji *et al.* (2007) of the presence of a major QTL on chromosome 2R conferring pyrerthoid

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resistance and containing a cluster of CYP6 genes including CYP6P9 supports this gene's association with pyrethroid resistance in *A. funestus*. If the upregulation of this enzyme is responsible for detoxification of pyrethriods the resistance mutation is likely to be a change in a promoter or *cis*-acting regulatory region.

Experimental procedures

Biological material

Two laboratory colonies of *A. funestus* were used in this study: FANG originates from material collected in southern Angola and is susceptible to all insecticides (Hunt *et al.*, 2005). FUMOZ-R originates from material collected in southern Mozambique and has been selected for resistance to permethrin (Hunt *et al.*, 2005). These strains are kept at the Vector Control Reference Unit of the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. They are maintained in standard insectary environment of 25 °C with 80% relative humidity and 12 h day/night, 45 min dusk/dawn lighting cycle.

WHO insecticide susceptibility assays

Newly emerged FUMOZ-R male and female adult mosquitoes were separated into cages containing a 10% sugar solution. Ten replicates of 25–30 adult mosquitoes each, at either day 3 or 14 after emergence, were exposed to 0.75% permethrin treated papers for 1 h according to the standard WHO bioassay protocol (WHO, 1998). The 60 min knock-down and final mortalities 24 h post exposure were recorded.

Insecticide dose-response analysis

Cohorts of 3–5 day old adult females were collected from the FUMOZ-R and FANG strains and exposed to a series of permethrin concentrations using a method based on the CDC bottle bioassay procedure of Brogdon & McAllister (1998). Sets of 250 ml treated bottles were produced containing the following series of permethrin concentrations (µg active ingredient per 250 ml bottle): 0.1 µg; 1 µg; 10 µg; 25 µg; 50 µg; 100 µg; 250 µg; 500 µg and 1000 µg. Exposures at each concentration lasted 1 h. 25–30 females were exposed per bottle through 8 replicates per set of bottles. Final mortalities 24 h post exposure at each concentration was recorded. The results were converted to a logarithmic scale and the 50% lethal dose (LD_{50}) was calculated for each cohort. Mean LD_{50} values were compared between the FUMOZ-R and FANG strains using a 2 sample *t* test.

Cohorts of 4th instar larvae were collected from the FUMOZ-R and FANG strains and exposed to a series of permethrin concentrations according to the standard WHO method (WHO, 2005). Sets of bowls containing 100 ml distilled water were treated with permethrin using the following range of concentrations: 990 µg/ml; 19.4 µg/ml; 9.7 µg/ml; 0.37 µg/ml; 3.6 ng/ml. Exposures at each concentration lasted 24 h. 25–30 larvae were exposed per bowl through 5 replicates for FANG and 6 replicates for FUMOZ-R per set of bowls. Final mortalities 2 h post exposure at each concentration was recorded. The results were converted to a logarithmic scale and the 50% lethal concentration (LC_{50}) was calculated for each cohort. Mean LC_{50} values were compared between the FUMOZ-R and FANG strains using a 2 sample t test.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from ten three-day-old adult FUMOZ-R and FANG strains of *A. funestus* using Tri-reagent (Sigma, MO, USA). The RNA was deoxyribonuclease (DNAse)-treated. RNA extractions were repeated on the treated samples and the pellets were re-suspended in 20 µl of DEPC-treated water. The extracted RNA was quantified using a GeneQuant *pro* Spectrophotometer (semi-quantitative PCR) or NanoDrop® Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored in aliquots (5 µl) at −70 °C. To prepare cDNA, total RNA was primed with 500 µg oligo (dT) adaptor (5′-GACTCGAGTCGACATCGA-3′) (Amenya *et al.*, 2005) and reverse transcribed using Superscript III (Invitrogen, CA, USA) according to manufacturer's instructions.

Dot blot analysis

Dot blot analysis was carried out in order to identify the family of P450 that was over expressed in the resistant strain FUMOZ-R. Serial dilutions of RNA from both FUMOZ-R and FANG strains (from 25 µg to 0.049 µg) were spotted onto BioBond™- plus nylon membrane (Sigma, USA). The membranes were UV cross linked and hybridised using DNA probes synthesised by PCR and labelled with DIG according to manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). The templates used for preparing DIG probes were from plasmids containing the genes of interest and were confirmed by sequencing (Amenya *et al.*, 2005). Each gene was separately labelled with DIG followed by denaturing the labelled probe (25 ng) with 1 ml of hybridisation solution (Roche Diagnostics). Cocktail probes were prepared in a final volume of 10 ml hybridisation solution. Hybridisation was carried out independently with either one of the cocktails of probes for CYP4, CYP6 or CYP9 genes. The CYP4 cocktail probe consisted of 12 genes (CYP4C25, CYP4C41, CYP4C40, CYP4C36, CYP4D26, CYP4D27, CYP4D25, CYP4G21, CYP4H14, CYP4J11, CYP4J9 and CYP4J12). The CYP6 cocktail probe consisted of 6 genes (CYP6P1, CYP6P2, CYP6P9, CYP6Z1, CYP6M8 and CYP6M7) while the CYP9 cocktail contained 5 genes (CYP9J12, CYP9J13, CYP9J11, CYP9L2/L3 and CYP9M3). After hybridisation, membranes were washed twice in low stringency wash solution (2 × SSC, 0.1% SDS) at room temperature for 5 min and washed twice at high stringency (0.1 × SSC, 0.1% SDS) at 68 °C for 15 min. After washes, the anti-DIG antibody was used to bind to the labelled probes and antibody binding visualised using chemiluminescent substrate, CSPD (Roche Diagnostics). Each membrane was mounted onto an exposure cassette and a Kodak® Bio Max light film, Light-1 (Kodak, FL, U.S.A.) was placed on top and developed.

To determine the specific gene that was over expressed, FUMOZ-R and FANG, RNA were serially diluted from 50 µg to 0.098 µg and probed with individual CYP6 genes. The level of expression of each gene was compared between the two mosquito strains.

A cDNA coding for the ribosomal protein S7 (rsp7) (Salazar *et al.*, 1993) was cloned from *A. funestus* and sequenced (Accession number EF450776). The plasmid containing the partial fragment of rsp7 was DIG labelled and used as an internal standard for RNA loading in dot blot and northern blot analysis. The membranes were stripped and re-hybridised using the rsp7 probe. The dot blots were repeated four times with independent RNA preparations.

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Northern blot analysis

Over expression of CYP6P9 was validated using northern blot analysis. The CYP6P9 probe was used to probe RNA on the membrane. RNA (5 µg) was electrophoresed on a 1.2% formaldehyde agarose gel and transferred by capillary action as described by Sambrook *et al.*, (1989). The membrane was hybridised as described in dot blots with the rsp7 gene used as an internal standard.

Quantitative Real-Time PCR (Q-PCR)

Q-PCR was used to validate the northern analysis. Products were sequenced to confirm the identity of the amplified products. The sequence of the CYP6P9 from both strains was deposited in genbank (Accession numbers FANG: EF152577, FUMOZ-R: DQ324779.

Primer design

The full-length CYP6P9 gene sequence (Matambo *et al.*, unpublished data) was used to design the specific primer using the Beacon Designer 3.0 software (Biorad, Hercules, CA, USA). The primer sequences are as follows: Cyp6real Fwd1 (5′-GAGGAAGTGAAGAAGCGACATC-3′) and Cyp6real Rev1, 5′-TGACGGTGAGAAGCGGAAC-3′). Rsp7 (EF450776) was used as a reference gene to normalise data (primer sequence as described above) and annealed to both FUMOZ-R and FANG.

Quantification of CYP6P9 expression using real-time PCR

The fold expression of CYP6P9 was quantified in triplicates using the iCycler IQ™ (Biorad, Hercules, CA, USA). RNA and cDNA synthesis for adults and early life stages was prepared as describe above. Each 25 µl reaction contained 12.5 µl SYBR Green supermix (Biorad) and 4 µl of both primers (25 µM), 2.5 µl cDNA (60–80 ng). The PCR program consisted of an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s. Data was collected during each extension cycle. This was followed by an auto-extension cycle of 72 °C for 4 min. The last step included a melting curve analysis. A standard curve was generated using 2-fold serial dilutions of cDNA (1 µg to 0.15 µg). The slope of the standard curve was used to validate the curve and those samples that did not attain the recommended slope of 3.3–3.8 and PCR efficiency of 90–105% were discarded (http://www.Qiagen.com). Fold over expression of CYP6P9 was calculated using the method described by Pfaffl (Pfaffl, 2001). Student *t*-test was used to validate significance of fold over expression.

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Sub-lethal pyrethroid exposure at the larval or adult life stage and selection for resistance in the major African malaria vector *Anopheles funestus* (Diptera: Culicidae)

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Malaria vector control by indoor residual spraying (IRS) in South Africa currently relies on the use of DDT to control pyrethroid-resistant populations of the major malaria vector *Anopheles funestus*. Pyrethroid resistance in *An. funestus* in South Africa and Mozambique is primarily based on the over-production of monooxygenase P450 genes. Preliminary evidence suggests that the pyrethroid-resistant phenotype is primarily expressed in adults, raising questions as to how resistance was selected for in the field given that there was no malaria control targeting adults in southern Mozambique when this resistant phenotype was first discovered there. The aim of this study therefore was to select for pyrethroid resistance at the larval or adult stages in southern African *An. funestus* and then assay the pyrethroid susceptibility of subsequent life stages using samples of progeny from the pyrethroid-resistant selections. There was no significant variation between the lethal permethrin concentrations inducing 50 % mortality (LC_{50}s) of larvae from three *An. funestus* colonies, but highly significant variation in the lethal dosages inducing 50 % mortality (LD_{50}s) of adults. It was not possible to enhance adult resistance/tolerance to permethrin by one round of selection at either the larval or adult stages in any of the colonies, although larval tolerance to permethrin can be enhanced by selection at the larval stage. It is concluded that monooxygenase-based pyrethroid resistance in southern African *An. funestus* is primarily expressed in the adult stage and that this particular resistance haplotype can only be selected by exposing adult mosquitoes to pyrethroids. This has important implications for malaria vector control in southern Africa because it suggests that the pyrethroid resistance in *An. funestus* populations in South Africa and southern Mozambique was selected for by private pyrethroid use directed against adult mosquitoes.

Key words: *Anopheles funestus*, life stage, malaria vector control, pyrethroid resistance, selection.

INTRODUCTION

Malaria vector control in South Africa was first implemented in response to a malaria outbreak in the province of KwaZulu-Natal during 1931/1932. This intervention was based on indoor spraying using a pyrethrum solution in kerosene. DDT was first used for malaria vector control in 1946 and was used continuously until synthetic pyrethroid insecticides were introduced for the same purpose in 1996 (Coetzee & Hunt 2000). Subsequently, the number of malaria cases recorded in South Africa increased steadily, leading to an epidemic that peaked in 2000, with 64000+ recorded cases (Govere *et al.* 2002; Coetzee 2005). Entomological investigations in KwaZulu-Natal revealed the unexpected presence of the major malaria vector *Anopheles funestus* Giles (Diptera: Culicidae) (Hargreaves *et al.* 2000). This species had previously been eliminated from South Africa's malaria-affected regions as a consequence of the use of DDT. The reoccurrence of *An. funestus* in KwaZulu-Natal was linked to the development of pyrethroid resistance, based on the postulate that pyrethroid-resistant *An. funestus* in the neighbouring Beluluane region of southern Mozambique had expanded its range into KwaZulu-Natal following the replacement of DDT with pyrethroids (Hargreaves *et al.* 2000; Brooke *et al.* 2001; Coetzee 2005). Consequently, DDT was reintroduced for malaria vector control in South Africa in conjunction

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with pyrethroids, and the number of recorded malaria cases has subsequently decreased to pre-1996 levels (Coetzee 2005; Maharaj *et al*. 2005).

Anopheles funestus is the nominal member of the *An. funestus* species group and is the only member implicated as a major vector of malaria. It is almost entirely endophilic (indoor-resting), endophagic (indoor feeding) and anthropophilic (human feeding) (Gillies & Coetzee 1987). As a consequence of endophily, *An. funestus* is particularly susceptible to control by indoor residual spraying (IRS), assuming the insecticide in use retains its efficacy. The development of pyrethroid resistance in *An. funestus* in South Africa and southern Mozambique effectively reduced the efficacy of this class of insecticide in the affected regions, providing an important example of how insecticide resistance can undermine the effectiveness of a malaria control programme based on IRS.

Pyrethroid resistance in southern African *An. funestus* adults is based on the over-production of monooxygenase P450 genes. The oxidizing enzyme products of these genes recognize pyrethroids as substrates and are able to detoxify them. When these monooxygenases are over-produced at a level sufficient to prevent internalized pyrethroid from reaching the neuronal target site, a pyrethroid-resistant phenotype is produced (Brooke *et al*. 2001; Amenya *et al*. 2005; Amenya *et al*. 2008; Wondji *et al*. 2009; Christian *et al*. 2011, Irving *et al*. 2012). The pyrethroid-resistant phenotype is also linked to thickened cuticles in adults which is likely to enhance the efficiency of P450-mediated detoxification by slowing the rate on insecticide absorption through the cuticle (Wood *et al*. 2010). However, one of the P450's implicated in resistance in adults, *CYP6P9a*, is not up-regulated in larvae (Amenya *et al*. 2008; Christian 2012). Furthermore, a comparison of pyrethroid susceptibility in samples of larvae drawn from an insecticide-susceptible *An. funestus* laboratory strain (FANG) and a strain selected for resistance to pyrethroids (FUMOZ-R) showed only a marginal decrease in susceptibility to permethrin in FUMOZ-R (Amenya *et al*. 2008). These data suggest that pyrethroid resistance in southern African *An. funestus* is primarily expressed in the adult stage, raising questions as to how resistance was selected for in the field given that there was no malaria control targeting adults in southern Mozambique prior to and during the 1996–2000 malaria epidemic in South Africa.

The aim of this study was to select for resistance to pyrethroid at the larval or adult stages of southern African *An. funestus* and then assay the pyrethroid susceptibility of subsequent life stages using samples of the progeny of survivors from the pyrethroid selections.

MATERIAL AND METHODS

Mosquito samples

Samples were drawn from three *An. funestus* laboratory colonies: FANG originates from material collected in southern Angola, and is fully susceptible to all insecticides; FUMOZ was reared from material collected in the Beluluane region of southern Mozambique and is partially resistant to pyrethroids; FUMOZ-R is a highly permethrin-resistant strain that was selected from FUMOZ (Hunt *et al*. 2005). These colonies are maintained under standard insectary conditions at the National Institute for Communicable Diseases in Johannesburg (Hunt *et al*. 2005).

Determination of the baseline levels of susceptibility to permethrin in the larval and adult life stages of laboratory reared An. funestus

Larvae and adults from each of the above colonies were exposed to a series of permethrin concentrations in order to determine LC_{50} and LD_{50} values (Lethal Concentration/Dose inducing 50 % mortality) (adapted from: Brogdon & McAllister 1998; Centers for Disease Control and Prevention 2002; WHO 1998). Quantification of susceptibility/resistance in this way enabled direct comparisons between samples as well as the determination of appropriate insecticide concentrations for subsequent resistance selections and resistance assessments.

Larvae

In order to determine LC_{50}s for larvae from each of the *An. funestus* strains, 25 fourth instar larvae were transferred to each of a series of polystyrene cups containing 80 ml distilled water. The larvae were left to acclimatize for at least one hour. One ml of acetone solution containing the desired concentration of permethrin (analytical grade, Sigma) was added to each cup, which was then topped up to 100 ml with more distilled water. The water in each cup was gently agitated to ensure a homogenous insecticide solution. The concentra-

tion range chosen (1×10^{-6}; 1×10^{-5}; 1×10^{-4}; 5×10^{-3}; 1×10^{-3}; 1×10^{-2} μg/ml) was sufficient to induce a range of mortalities from 0 % to 100 % for each of the *An. funestus* colonies. Exposure to each concentration was replicated eight times per colony. Controls consisted of larvae in cups containing 100 ml distilled water treated with 1 ml acetone.

Following insecticide exposure for 24 hours, each cup was gently drained through a strainer. The larvae in the strainer were washed twice with distilled water and excess water was removed by blotting the strainer with tissue paper. The larvae were then washed into recovery cups containing approximately 80 ml fresh distilled water, subsequently topped up to 100 ml.

Mortality was scored following a two-hour recovery period. There were some complications when scoring larval mortality because dead larvae do not necessarily sink. Furthermore, live *An. funestus* larvae can remain submerged for long periods, and can be confused with dead larvae. Therefore, the shaft of a dissecting needle was used to gently prod and submerge any floating larvae. Those larvae that did not resurface after prodding after 10 minutes were scored as dead. The LC_{50}s for each colony were calculated using log transformations and probit analysis of the mean mortalities from each concentration.

Adults

Permethrin-coated 250 ml glass bottles (Brogdon & McAllister 1998) were used to determine LD_{50}s for adults from each of the *An. funestus* strains. The bottles were coated with 1 ml of permethrin-acetone solution at the concentrations of 1, 5, 25, 50; 100 and 250 μg/ml/bottle. Control bottles were prepared using 1 ml of acetone only per bottle. Coated bottles were prepared at least 48 hours prior to use. Bottles awaiting use were stored in the dark at room temperature. Each bottle was only used twice.

To conduct the bioassays, 25 females were gently aspirated into each coated bottle. Exposures were conducted for one hour following which all mosquitoes were removed and placed into appropriately labelled non-treated containers. A wad of cotton wool soaked in a 10 % sucrose solution was placed on top of each container immediately post-exposure and was left for the duration of the experiment to allow survivors to feed on the sucrose. Final mortalities were scored 24 hours post-exposure and the LD_{50}s for each colony were calculated using log transformations and probit analysis of the means from each concentration. Exposure to each dose or control was replicated five times per colony.

Assessment of permethrin resistance following selection for resistance at the larval stage

In order to select for resistance to permethrin at the larval stage, between 1000 and 2000 fourth instar larvae were placed in a container with distilled water. Samples of larvae from each colony were also used to determine their resistance levels against the LC_{50} previously determined for each colony. These were denoted as L_B (baseline generation – B; assessed for resistance at the larval stage – L).

Each container was treated with a permethrin solution at the required LC_{50} for each colony. After 24 hours exposure, all larvae from each treatment were removed by straining through gauze netting, washed and transferred to a clean bowl with fresh distilled water. Surviving larvae were reared through to pupae and emerging adults were pooled into mating cages by colony. Each cage contained a vial of cotton wool soaked in a 10 % sucrose solution. These were denoted F0LA (parental generation – F0; selected at the larval stage – L; assessed for resistance at the subsequent adult stage – A). Samples of F0LA adult females were used to determine their levels of resistance to permethrin at their respective LD_{50}s following selection at the larval stage. The remainder were given blood meals in order to produce eggs. Their progeny were labelled as F1LL (Progeny of parental generation – F1; permethrin selected at the larval stage – L; assessed for permethrin resistance at the subsequent larval stage – L).

The F1LL larvae were reared to fourth instar and each colony was assessed for their level of resistance to their respective baseline LC_{50}s. The remainder were reared to adulthood and denoted as F1LA (Progeny of parental generation – F1; permethrin selected at the larval stage – L; assessed for permethrin resistance at the subsequent adult stage – A). Samples of F1LA adult females were used to determine their resistance levels at their respective baseline LD_{50}s.

Assessment of permethrin resistance following selection for resistance at the adult stage

Exposure of adults to permethrin was performed using bottles coated internally with the appropriate LD_{50} concentration. A large number of 2–3-day-

old adults (both male and female) were drawn from the same cohort of each colony and placed in separate cages where only sugar water was available. Samples of males and females (25 per bottle) were then drawn for five exposure replicates. All exposures were conducted as previously described. This cohort was denoted A-B (baseline generation – B; assessed for resistance at the subsequent adult stage – A). Following exposure all mosquitoes were removed and placed in containers in order to obtain the final mortalities 24 hours post-exposure. Survivors were then removed from their recovery containers and placed in mating cages by colony. They were then blood-fed and encouraged to lay as many eggs as possible. Their progeny were designated F1AL (Progeny of parental generation – F1; permethrin selected at the adult stage – A; assessed for permethrin resistance at the subsequent larval stage – L). These were reared to fourth instar and samples were assayed against their appropriate colony LC_{50}s to determine their resistance levels based on mortalities. The remainder, denoted as F1AA (Progeny of parental generation – F1; permethrin selected at the adult stage – A; assessed for permethrin resistance at the subsequent adult stage – A), were reared to adulthood and assayed against their appropriate LD_{50}s in order to determine their levels of permethrin resistance by colony, based on mortalities 24 hours post-exposure.

Data analysis

Variations in LC_{50}s in larvae and LD_{50}s in adults by replicate were compared between FANG, FUMOZ and FUMOZ-R using one-way ANOVA. Comparisons of mortalities following exposure to permethrin at previously established LC_{50} (larvae) or LD_{50} (adults) between the baseline parental strains (A_B and L_B) and resistance-selected generations (F0LA, F1LL, F1LA, F1AL, F1AA) by colony by replicate were based on one-way ANOVA. The proportional LD_{50} increase in FUMOZ-R over FANG was calculated by dividing the mean FUMOZ-R LD_{50} by that obtained for FANG.

RESULTS

The LC_{50}s derived from pyrethroid exposed larvae and the LD_{50}s derived from pyrethroid exposed adult females are given in Fig. 1. There was no significant variation in LC_{50} between

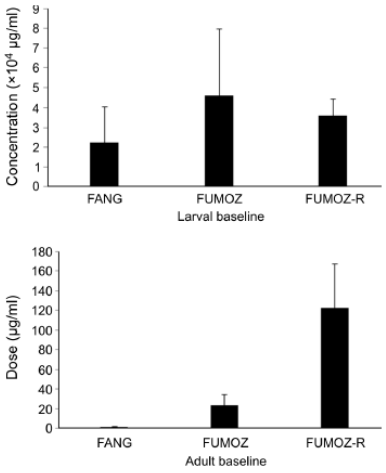

Fig. 1. Mean lethal permethrin concentrations inducing 50 % mortality (LC_{50}) for larvae (**top**) and mean lethal dosages inducing 50 % mortality (LD_{50}) for adult females (**bottom**) from the *Anopheles funestus* FANG (FNG), FUMOZ (FMZ) and FUMOZ-R (FMZR) laboratory colonies. Standard errors shown.

FANG, FUMOZ and FUMOZ-R larvae (ANOVA: d.f. = 2, $F = 1.93$, $P = 0.17$). However, the variation in LD_{50} between adult females from each colony was highly significant (ANOVA: d.f. = 2, $F = 68.1$, $P < 0.01$) with FANG giving the lowest LD_{50} and FUMOZ-R the highest. There was a 76.1-fold increase in the FUMOZ-R LD_{50} over FANG.

Assessments of permethrin resistance following selection for resistance at the larval and adult stages are summarized in Fig. 2. Selection for resistance at the larval stage induced a significantly increased permethrin tolerance in larvae of the subsequent generation in FANG (ANOVA: d.f. = 1, $F = 85.3$, $P < 0.05$) and FUMOZ-R (ANOVA: d.f. = 1, $F = 43.3$, $P < 0.05$). This trend was not apparent in FUMOZ (ANOVA: d.f. = 1, $F = 8.7$, $P > 0.05$). This same selection significantly decreased permethrin tolerance in adults of the subsequent generation in FANG and FUMOZ (ANOVA – FANG: d.f. = 1, $F = 12.7$, $P < 0.05$, ANOVA – FUMOZ: d.f. = 1, $F = 17.5$, $P < 0.05$) but not in FUMOZ-R (ANOVA: d.f. = 1, $F = 1.62$, $P = 0.23$).

Selection for resistance at the adult stage did

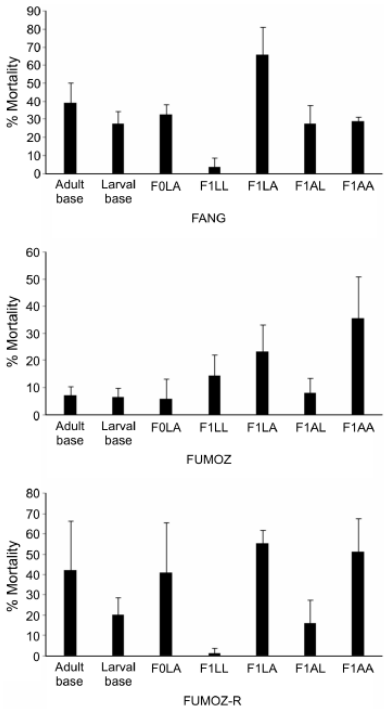

Fig. 2. Mean percentage mortalities for samples of either adults or larvae drawn from three *Anopheles funestus* laboratory colonies (FANG, FUMOZ, FUMOZ-R) following selection with and subsequent exposure to their respective permethrin LD/LC_{50}s. F0LA = parental generation – F_0; permethrin selected at the larval stage – L; assessed for permethrin resistance at the subsequent adult stage – A. F1LL = progeny of parental generation – F_1; permethrin selected at the larval stage – L; assessed for permethrin resistance at the subsequent larval stage – L. F1LA = progeny of parental generation – F_1; permethrin selected at the larval stage – L; assessed for permethrin resistance at the subsequent adult stage – A. F1AL = progeny of parental generation – F_1; permethrin selected at the adult stage – A; assessed for permethrin resistance at the subsequent larval stage – L. F1AA = progeny of parental generation – F_1; permethrin selected at the adult stage – A; assessed for permethrin resistance at the subsequent adult stage – A. Standard errors shown.

not affect permethrin tolerance in the larvae of subsequent generations in any of the colonies (ANOVA – FANG: d.f. = 1, $F = 3.16$, $P = 0.11$); (ANOVA – FUMOZ: d.f. = 1, $F = 0.16$, $P = 0.69$); (ANOVA – FUMOZ-R: d.f. = 1, $F = 7.17$, $P > 0.05$). It also did not affect permethrin tolerance in the adults of subsequent generations in FANG (ANOVA:d.f. = 1, $F = 3.36$, $P = 0.1$) and FUMOZ-R (ANOVA: d.f. = 1, $F = 0.44$, $P = 0.53$), whilst permethrin tolerance in FUMOZ significantly decreased following selection in adults of the previous generation (ANOVA: d.f. = 1, $F = 24.7$, $P < 0.05$).

DISCUSSION

The highly significant variation in permethrin tolerance between adults of FANG, FUMOZ and FUMOZ-R was not apparent in the larvae of each strain. Despite the measurable occurrence of permethrin resistance in adult FUMOZ and FUMOZ-R compared to the insecticide-susceptible FANG, the larval response to permethrin exposure between all three colonies was comparable, showing that the permethrin-resistance phenotype in FUMOZ and FUMOZ-R was only expressed in the adult stage.

Based on the assessments of permethrin resistance following selection at the larval and adult stages, it was not possible to enhance adult resistance/tolerance by one round of selection in any of the *An. funestus* colonies. This may have occurred because FANG does not carry selectable permethrin resistance alleles (Christian *et al*. 2011) whilst permethrin resistance in FUMOZ-R may be fixed (Hunt *et al*. 2005; Wondji *et al*. 2009), precluding the possibility of enhancing resistance by selection of the adults of this colony any further. The measurable decrease in permethrin resistance in FUMOZ adults following selection in adults of the previous generation is an anomaly that cannot be readily explained, but may be an inadvertent consequence of selection for low-level polygenic resistance using a sub-lethal dosage (LD_{50}). The data for FANG and FUMOZ-R showed that larval tolerance to permethrin can be enhanced by selection at the larval stage of the previous generation, although this effect was not apparent in FUMOZ. Most importantly, these data showed that selection for permethrin resistance/tolerance at the larval stage reduced permethrin tolerance in adults of the subsequent generation in FANG and FUMOZ.

The absence of measurable permethrin resistance phenotypes in FUMOZ and FUMOZ-R larvae despite the occurrence of the resistance phenotype in adults from the same colonies is in accord with the molecular data of Amenya *et al*. (2008) which showed that the pyrethroid resistance associated *CYP6P9* is only up-regulated in FUMOZ-R adults. It thus follows that selection for pyrethroid resistance in FUMOZ and FUMOZ-R, and thereby the wild *An. funestus* population from which they were derived, most likely occurred at the adult stage as opposed to the larval stage. This is important because insecticide resistance in vector populations often occurs at the larval stage as a consequence of the leaching of insecticide into their breeding sites following insecticide application for agricultural pest control purposes (van der Werf 1998). Yet the data presented here for southern African *An. funestus* suggest that permethrin selection at the larval stage is likely to suppress the occurrence of resistance in adults. Therefore, the occurrence of pyrethroid resistance in southern African *An. funestus* was likely selected as a consequence of exposure of adult mosquitoes to pyrethroid insecticides.

It is concluded that monooxygenase-based pyrethroid resistance in southern African *An. funestus* is primarily expressed in the adult stage and that selection for this particular resistant haplotype can only be done by exposing adult mosquitoes to pyrethroids. This has important implications for malaria vector control in southern Africa because it suggests that the pyrethroid resistance recorded in *An. funestus* populations in South Africa and southern Mozambique was selected by private insecticide use directed against adult mosquitoes.

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APPENDIX B: PUBLICATION RESULTING FROM CHAPTER 4

Contribution to publication:

Oliver Wood developed the technique described in the publication for ready mosquito leg cuticle thickness assessment using a scanning electron microscope the cuticle thickness of mosquito legs, and performed and analysed all of the laboratory work and resulting data, and compiled the initial drafts of the manuscript.

Wood et al. Parasites & Vectors 2010, 3:67
http://www.parasitesandvectors.com/content/3/1/67

Parasites & Vectors

RESEARCH Open Access

Cuticle thickening associated with pyrethroid resistance in the major malaria vector *Anopheles funestus*

OR Wood[1,2], S Hanrahan[3], M Coetzee[1,2], LL Koekemoer[1,2], BD Brooke[1,2*]

Abstract

Background: Malaria in South Africa is primarily transmitted by *Anopheles funestus* Giles. Resistance to pyrethroid insecticides in *An. funestus* in northern Kwazulu/Natal, South Africa, and in neighbouring areas of southern Mozambique enabled populations of this species to increase their ranges into areas where pyrethroids were being exclusively used for malaria control. Pyrethroid resistance in southern African *An. funestus* is primarily conferred by monooxygenase enzyme metabolism. However, selection for this resistance mechanism is likely to have occurred in conjunction with other factors that improve production of the resistance phenotype. A strong candidate is cuticle thickening. This is because thicker cuticles lead to slower rates of insecticide absorption, which is likely to increase the efficiency of metabolic detoxification.

Results: Measures of mean cuticle thickness in laboratory samples of female *An. funestus* were obtained using scanning electron microscopy (SEM). These females were drawn from a laboratory colony carrying the pyrethroid resistance phenotype at a stable rate, but not fixed. Prior to cuticle thickness measurements, these samples were characterised as either more or less tolerant to permethrin exposure in one experiment, and either permethrin resistant or susceptible in another experiment. There was a significant and positive correlation between mean cuticle thickness and time to knock down during exposure to permethrin. Mean cuticle thickness was significantly greater in those samples characterised either as more tolerant or resistant to permethrin exposure compared to those characterised as either less tolerant or permethrin susceptible. Further, insecticide susceptible female *An. funestus* have thicker cuticles than their male counterparts.

Conclusion: Pyrethroid tolerant or resistant *An. funestus* females are likely to have thicker cuticles than less tolerant or susceptible females, and females generally have thicker cuticles than males. In pyrethroid resistant *An. funestus*, this increase in cuticle thickness is likely to have developed as an auxiliary to the primary mode of pyrethroid resistance which is based on enzyme-mediated detoxification.

Background

Malaria in southern Africa is transmitted by *Anopheles funestus* Giles, the nominal member of the *An. funestus* species group, and *An. arabiensis* Patton, a member of the *An. gambiae* species complex.

Malaria vector control in South Africa and many of its neighbouring countries is based on the application of insecticides inside human dwellings and other structures in affected areas. The effectiveness of this approach hinges on production issues such as insecticide formulation, logistical issues such as adequate and timely coverage, and biological issues of which the emergence of insecticide resistance in target mosquito populations is the most pressing.

Resistance to pyrethroid insecticides in *An. funestus* in northern Kwazulu/Natal, South Africa, and in neighbouring areas of southern Mozambique enabled populations of this species to increase their ranges into areas where pyrethroids were being exclusively used for malaria control [1-3]. The effect of this range expansion, as well as the emergence of anti-malarial drug resistance, was an

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unprecedented malaria epidemic, primarily in Kwazulu/Natal, South Africa, during the period 1996 to 2000. Adequate responses including the re-introduction of DDT and a change in the prescribed anti-malarial drug regimen succeeded in controlling the epidemic [3,4]. Nevertheless, this incident highlighted the potential effect that insecticide resistance can have on an otherwise well-implemented vector control programme.

Pyrethroid resistance in southern African *An. funestus* is primarily based on an enzyme system that employs the up-regulated detoxifying capabilities of at least two P450 monooxygenase genes [2,5-7], with variation in expression of the resistance phenotype by age and gender [8]. It is likely that resistance in *An. funestus* arose comparatively rapidly as a consequence of intense insecticide selection pressure [1]. As a result, other factors may be directly or indirectly involved in the expression of the resistance phenotype. One possibility is cuticular thickening. Thicker cuticles lead to slower rates of insecticide absorption, which is likely to enhance the efficiency of metabolic detoxification. Slower insecticide penetration across the cuticle (though not necessarily the result of cuticle thickening) has been associated with insecticide resistance in the cotton bollworm *Helicoverpa armigera* [9,10].

Cuticle thickening is a gene-regulated process that an insect undergoes as it ages. It has been demonstrated that cuticle is laid down in a circadian pattern, resulting in growth rings of nocturnal lamellate and diurnal non-lamellate structure [11-13]. The periods of transcription of genes coding for cuticle formation have been used to predict age in the dengue vector *Aedes aegypti* in the field [14], and aging by quantification of cuticle rings has been described for African migratory locusts [15].

Measurable cuticle thickening has been associated with pyrethroid resistance in the Chagas disease vector *Triatoma infestans* [16] and is tentatively inferred from micro-array gene transcription analysis in *An. stephensi* [17]. Measurements of rates of insecticide penetration have been found to be affected by thickened cuticles as well as by other structural components of cuticles such as relative amounts of surface hydrocarbons [16], suggesting that decreased rates of penetration across the cuticle slows insecticide inoculation of internal organs sufficiently to allow for effective metabolically-mediated detoxification.

In a preliminary micro-array analysis of pyrethroid resistant and susceptible *An. funestus* [unpublished data] using the *An. gambiae* detoxification chip [18], differential transcription of a gene associated with cuticle deposition (JV2) was observed. This provided the impetus to compare cuticle thicknesses between laboratory reared *An. funestus* samples characterised by their responses to pyrethroid intoxication. A direct measure of association between these two phenotypes (response to insecticide intoxication and cuticle thickness) provides a critical platform for establishing whether they share a causal relationship, as well as for predicting the potential biological and epidemiological implications of such a relationship.

Methods

Permethrin tolerance assay

Anopheles funestus samples

Samples of *An. funestus* were drawn from the FUMOZ laboratory colony housed at the VCRU, NICD, in Johannesburg. This colony originates from material collected in southern Mozambique and is maintained under standard insectary conditions [8]. FUMOZ carries resistance to the pyrethroid insecticide permethrin at comparatively stable rates ranging between 5% and 30% mortalities [19], as measured using standard insecticide exposure assays against adults [20]. For the gender comparison, samples of *An. funestus* were drawn from the FANG laboratory colony. This colony originates from material collected in southern Angola and is fully susceptible to insecticides.

Insecticide exposure assay

In order to choose an appropriate adult mosquito age for cuticle thickness measurements, the findings of Cook *et al.* [14] concerning the expression periods of cuticle gene orthologue Ae-8505 in *Aedes aegypti* were used as a guide. It was decided to use ten day old female mosquitoes all drawn from the same FUMOZ cohort. This age cohort was chosen in an effort to minimise variation in thickness due to age dependent gene expression (allowing for variation of approximately 24 h as mosquito samples were collected once per day) as well as to obtain cuticles at their thickest in terms of growth and repair. It has previously been established that FUMOZ females show appreciable levels of pyrethroid resistance at 10 days [8].

Twenty adult females were aspirated into a WHO exposure tube containing a 0.75% permethrin treated filter paper supplied by the World Health Organization (WHO). Knockdown of mosquitoes in the exposure tube was continuously monitored for one hour. In order to validate knockdown of an individual, the base directly under the mosquito was lightly tapped in order to ascertain whether it was a true knockdown. Any individual that was still able to fly was kept in the exposure tube until it became completely moribund, at which point it was removed using an aspirator carefully inserted into the tube. The time of removal was recorded. Those individuals that were still active after 60 minutes exposure were grouped as + 60 minutes. These samples were cold-terminated in a fridge at 4°C within five minutes of removal from the permethrin exposure tube, preventing

further cuticle formation in any of the mosquitoes taken for subsequent analysis. Females representing the earliest knock-down cohort were grouped and termed as permethrin intolerant (< 30 minutes to knock-down), whilst females with a knock-down time in excess of 40 minutes were grouped as permethrin tolerant. These time discriminators presented as natural limits for FUMOZ based on exposure observations and are not standardised for anophelines in general. This process was repeated until suitable samples (at least 10) of individuals from the intolerant and tolerant groupings had been collected.

Specimen preparation for SEM

All females were washed twice in 70% ethanol in order to clean them thoroughly. The legs were given a light brushing in the area of the desired section while under the ethanol. This was done to remove some of the scales that cover the legs in order to facilitate a cleaner cut. Tarsomere I on the left middle leg was severed at the midpoint in a drop of alcohol using a new platinum coated blade (Figures 1 &2). Following the sectioning, the leg was washed again to remove any debris which may obscure the view of the cuticle. Fresh ethanol was gently passed over the section to remove any debris. The leg was left attached to the body. Each mosquito was then placed ventral side up in a foam critical point drying container with the sectioned portion of the leg orientated vertically. The containers were then passed through 70%, 80%, 90% and 100% ethanol. Each step lasted at least two hours and was repeated twice. The final change was allowed to stand overnight, and the ethanol used was kept under a molecular sieve to ensure complete dehydration. The dried specimens were mounted ventral side up on stubs, ensuring vertical positioning of the sectioned part of the leg so as to enable a square measurement across the section from above.

Specimens were sputter coated with carbon and gold palladium and viewed using a Jeol JSM-840 scanning electron microscope. Micrographs obtained were digitized and examined using Zeiss AxioVision Release 4.6 software to measure the thickness of the cuticle (Figure 3). Measurements were made by tracing the outline of both the inner and outer circumference of the cuticle and measuring the shortest distance between the two at no fewer than 25 different, evenly distributed points with obvious aberrations such as scale beds excluded. A mean cuticle thickness per specimen was obtained in this way. The permethrin tolerant and intolerant groups were then compared using one-way ANOVA and a possible trend between cuticle thickness and time to knock-down was evaluated using linear regression (Statistix 7.0 software).

Permethrin susceptibility assay

Following the tolerance assays, another large sample of FUMOZ females were taken from a single cohort. In order to offset the effect of circadian cuticular lay-down the sample population was aged to nine days at which point a sub-sample was removed for insecticide exposure while the remainder were allowed to age one more day. The nine and ten day old groups were exposed to 0.75% permethrin for 1 hour according to the standard WHO protocol for testing adult anopheline susceptibility to insecticide [20]. Exposures of both sub-samples were conducted at the same time of day under the same conditions.

Those survivors from the day 9 exposure were removed on the 10th day following a 24 hour recovery period and were cold terminated as previously described. These survivors were characterised as 10-day-old permethrin resistant. Those females that succumbed to permethrin exposure during the day 10 exposure were characterised as 10-day-old permethrin susceptible.

In order to accommodate an effect of body size on cuticle thickness, the wing lengths of all females used in the permethrin resistance assay were measured. Wing length gives a good approximation of body size [21].

Specimen preparation for SEM was as described earlier. An FEI Quanta 400 E scanning electron microscope,



Figure 1 Illustration of the point of section on *An. funestus* tarsomere 1 (after Evans [24](*t1-t5* = five tarsal segments).

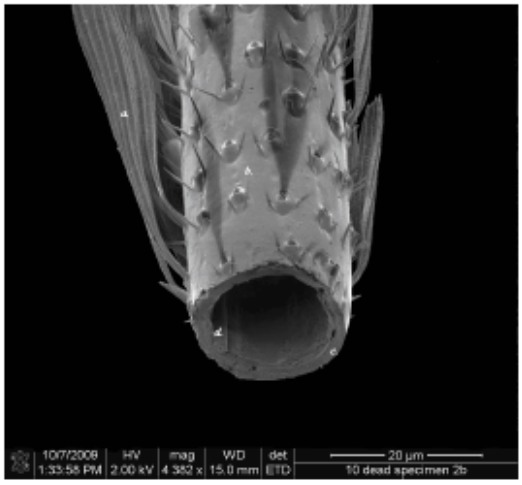

Figure 2 SEM (FEI Quanta 400 E) micrograph showing an oblique view of the lower half of a sectioned leg. a. Scale. b. Area where scales were brushed away to facilitate a clean section. c. The integument. d. Muscle or tendon (frequently used as a reference point for standardizing points of measure).

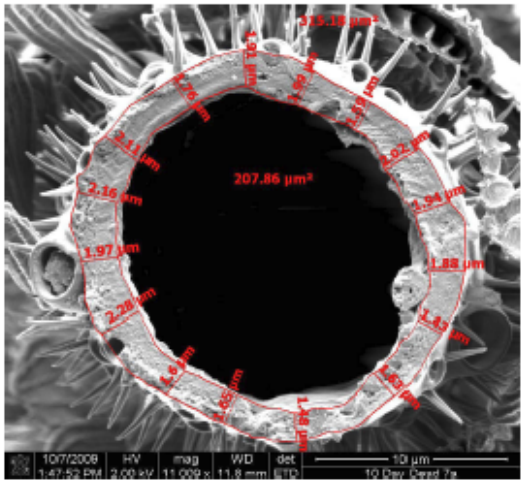

Figure 3 Micrograph from the FEI Quanta 400E illustrating 16 initial points of cuticle thickness measurement, allowing for calculation of mean cuticle thickness per individual. At least 25 points of measurements were used per individual.

which became available for use in the interim, was used to obtain digital micrographs. Mean cuticle thickness was compared between the permethrin resistant and susceptible samples using one-way ANOVA (Statistix 7.0 software).

Gender comparison

Given the variation in insecticide resistance phenotypic expression between males and females in southern African *An. funestus* (females are generally more tolerant/resistant [8]), it was decided to ascertain whether there is significant variation in cuticle thickness by gender. A sample of males and females was removed from a cohort of the FANG colony. This colony was used for this experiment because it does not carry any measurable insecticide resistance phenotypes, thus removing insecticide resistance as a confounding variable when comparing cuticle thickness between males and females. The sample was aged to five days before being cold terminated as in the previous assays. The wing lengths of all individuals used for subsequent cuticle measurements were determined.

All specimens were prepared for SEM as described earlier, and the FEI Quanta 400 E scanning electron microscope was used to obtain digital micrographs. The images on all micrographs were analysed and measured as described earlier and the mean cuticle thickness of each specimen was calculated. Mean cuticle thickness was compared between males and females using one-way ANOVA (Statistix 7.0 software).

Results

Permethrin tolerance assay

During viewing it was noted that a number of the prepared specimens had internal tissue protruding beyond the section edge, obscuring the cuticle. These specimens were discarded. In total, measurements of nine individuals from each of the pyrethroid intolerant and tolerant samples were obtained.

The mean cuticle thickness of the intolerant specimens was 2.13 µm (SD ± 0.10 µm) while the tolerant specimens showed a mean thickness of 2.33 µm (SD ± 0.22 µm), giving a mean difference of 0.20 µm (Figure 4). This difference is significant based on ANOVA (P = 0.03).

A linear regression of time to knock-down (kd_t) vs. mean cuticle thickness is shown in Figure 5. There is a significant trend (P = 0.01) in which cuticle thickness generally increases with increasing length of time to knock-down, although the correlation is weak (R^2 = 0.33).

Permethrin susceptibility assay

Measurements of mean cuticle thickness were obtained from 10 females characterised as 10-day-old permethrin resistant and 9 females characterised as 10-day-old permethrin susceptible. Only one specimen was discarded because of tissue obscuring the cuticle. There was no significant difference in wing-length measurements between the two samples (mean wing-length of resistant sample = 2.86 mm; mean wing-length of susceptible sample = 2.88 mm; P = 0.7084 based on a two sample t test).

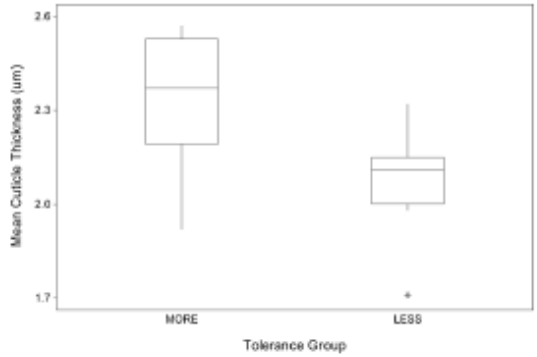

Figure 4 Means and variation in cuticle thicknesses (with 95% limits) of two samples of *An. funestus* laboratory reared females characterised as either more or less tolerant to permethrin intoxication.

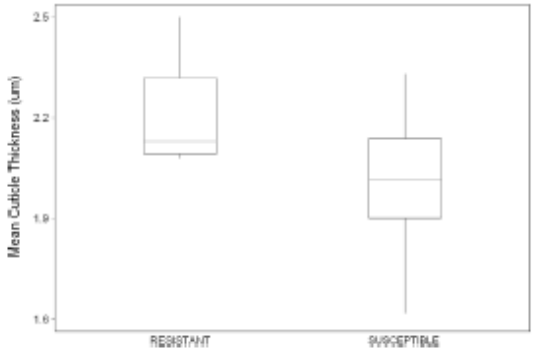

Figure 6 Means and variation in cuticle thicknesses (with 95% limits) of two samples of *An. funestus* laboratory reared females characterised as either resistant or susceptible to permethrin intoxication.

The mean cuticle thickness of the permethrin susceptible specimens was 2.00 μm (SD ± 0.20 μm) while the permethrin resistant specimens showed a mean thickness of 2.21 μm (SD ± 0.15 μm) (Figure 6), giving a mean difference of 0.21 μm. This difference is significant (P = 0.02) based on one-way ANOVA.

Gender comparison

One male specimen was discarded owing to tissue obscuring the cuticle, and measurements were obtained from 11 specimens. Two female specimens were discarded and cuticle measurements were obtained from 10 specimens. There was no significant variation in wing length between samples (mean wing-length of males = 3.01 mm; mean wing-length of females = 3.12 mm; P = 0.07 based on a 2 sample t test).

Mean cuticle thicknesses were 1.79 μm (SD ± 0.18 μm) for the male sample and 2.01 μm (SD ± 0.15 μm) for the female sample (Figure 7). The mean difference of 0.22 μm was significant based on ANOVA (P = 0.01).

Discussion

Insecticide resistant phenotypes and their underlying mechanisms tend to evolve rapidly under intense insecticide selection pressure, leading to the prediction that resistance, in general, is likely to be conferred by a small number of major-effect genes [22]. However, differential expression of many genes not normally associated with insecticide resistance may also occur [23].

Measurements of response to permethrin exposure, expressed either in terms of tolerance during exposure or outcome following exposure, show similar associations with mean cuticle thickness. There was a 9.5-10% increase in mean cuticle thickness in those individuals either more tolerant or resistant to permethrin compared

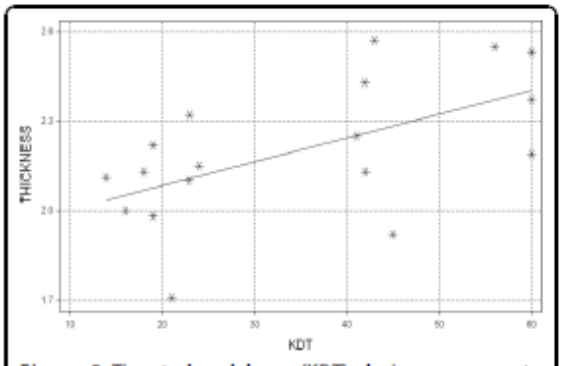

Figure 5 Time-to-knockdown (KDT) during exposure to permethrin vs. mean cuticle thickness (microns). The trend is significant but weakly correlated (R^2 = 0.33, P = 0.03).

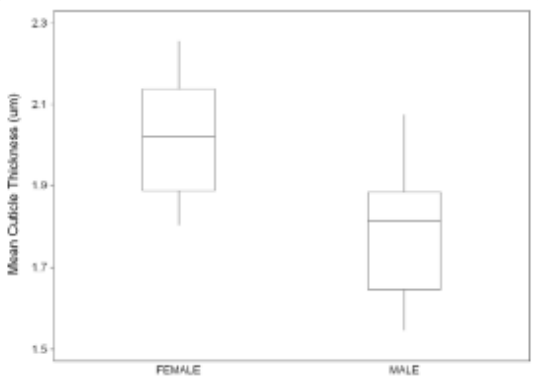

Figure 7 Means and variation in cuticle thicknesses (with 95% limits) of female and male *An. funestus* samples drawn from a laboratory colony that is fully susceptible to insecticide.

to those that were either less tolerant or permethrin susceptible. A link between cuticle thickness and response to insecticide exposure is thus based on the assortment of phenotypes, whereby the permethrin tolerant and resistant groups were significantly associated with thicker cuticles and vice versa. A similar difference in cuticle thickness was observed between insecticide susceptible males and females which may contribute to the differences in tolerance to insecticide intoxication generally observed between genders in *An. funestus* [8]. Variation in the expression of pyrethroid resistance with age in the FUMOZ colony is most likely based on variation in monooxygenase gene expression. Ten day old FUMOZ mosquitoes show reduced insecticide resistance compared to younger cohorts [8], but our data give no indication as to whether this variation is in any way linked to cuticle deposition.

These data support the hypothesis that the efficiency of monooxygenase-based pyrethroid resistance in southern African *An. funestus* is likely to be affected by minor factors. This is because in order to produce an effective resistant phenotype, upregulated transcription of selected P450 monooxygenase genes must produce sufficient enzyme to catalyze the metabolism of pyrethroids at a rate that prevents significant interaction between the insecticide and its neuronal target. Therefore, any mechanism that slows or regulates insecticide inoculation of internal organs, such as cuticular thickening, is a likely candidate for selection along with the primary mode of resistance.

Conclusion

We conclude that pyrethroid tolerant or resistant *An. funestus* females are likely to have thicker cuticles than less tolerant or susceptible females, and that females generally have thicker cuticles than males. In pyrethroid resistant *An. funestus*, this increase in cuticle thickness is likely to have developed as an auxiliary to the primary mode of resistance which is based on enzyme-mediated detoxification.

Insecticide resistance has the potential to undermine insecticide based vector control applications. Resistance management strategies, which aim to either circumvent or reduce the rate of insecticide resistance development in vector populations, are best served by as complete an understanding of resistance mechanisms and their potential effects as possible.

List of Abbreviations

SEM: Scanning Electron Microscopy; WHO: World Health Organisation; ANOVA: Analysis of Variance.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ORW assisted with experimental design, conducted the experiments, analysed the data and drafted the initial manuscript. SH assisted with experimental design and data interpretation, offered general expertise and advice, and reviewed the manuscript prior to submission. MC assisted with data interpretation and reviewed the manuscript prior to submission. LLK assisted with experimental design and reviewed the manuscript prior to submission. BDB assisted with experimental design, data analysis and interpretation, and produced the final version of the manuscript.

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