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BIOLOGY OF INSECTICIDE RESISTANCE IN THE AFRICAN MALARIA VECTOR *ANOPHELES FUNESTUS* (DIPTERA: CULICIDAE)

by

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

Johannesburg, 2007

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DECLARATION

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

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……. day of ……. 2007

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ABSTRACT

The emergence of pyrethroid resistant *Anopheles funestus* (a major African vector) in malaria affected parts of KwaZulu-Natal, South Africa was correlated with the malaria epidemic of 1996 - 2000. This finding prompted the necessity of incorporating insecticide resistance management strategies into formal malaria control policy in South Africa. Resistance management strategies often rely on the assumption of reduced fitness associated with insecticide resistance and are based on the principle that resistance genes will tend to drift out of vector populations in the absence of insecticide selection pressure. This study aimed to determine whether a fitness cost is associated with pyrethroid resistance as well as to determine the stability and mode of inheritance of the resistance genes in a pyrethroid resistant (FUMOZ-R) strain of *An. funestus*. It also aimed to sequence and analyze a segment of the sodium channel gene for any *kdr*-type mutation(s) that may be associated with pyrethroid resistance. The final aim was to determine the resistance mechanisms involved in a Ghanaian field population of *An. funestus* resistant to DDT and pyrethroids.

Results obtained suggest that pyrethroid resistance in southern African *An. funestus* did not incur any loss of fitness. FUMOZ-R had a reproductive advantage over a pyrethroid susceptible *An. funestus* strain (FANG) in terms of higher fertility, proportion of females laying eggs and egg-to-adult survivorship, and a lower sterility

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rate. However, FUMOZ-R had a slower developmental time from egg hatch to adult emergence than FANG.

Results of crosses and backcrosses carried out between FUMOZ-R and FANG were consistent with a monofactorial and autosomal mode of inheritance in which the resistant genes presented as incompletely dominant. The resistant gene was found to be stable over several generations in the absence of insecticide selection pressure.

Analysis of the genomic and mRNA sequences of the IIS5 - IIS6 segment of the sodium channel gene showed a high sequence identity between FUMOZ-R and FANG suggesting that the two strains are genetically similar. The *kdr*-type mutation was absent from this region supporting previous evidence that the resistance mechanism is primarily metabolic.

Bioassay data showed that a Ghanaian field population of *An. funestus* from Obuasi, Ghana, was resistant to DDT and pyrethroids. Molecular analysis of the IIS5 - IIS6 segment of the sodium channel gene showed an absence of *kdr*-type mutations previously associated with insecticide resistance. Biochemical analysis suggests that resistance is metabolically mediated primarily by elevated levels of α and β esterases with monooxygenases and GSTs playing a lesser role. The presence of an altered acetylcholinesterase conferring carbamate resistance was also evident in the population. These results have implications for the management of resistance in malaria control programmes in Africa.

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DEDICATION

This thesis is dedicated to my loving husband and my dear parents for all you have done for me.

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Publications and presentations based on this study

PUBLICATIONS:

Okoye, P.N., Brooke, B.D., Hunt R.H. & Coetzee M. (2007) Relative developmental and reproductive fitness associated with pyrethroid resistance in the major southern African malaria vector *Anopheles funestus*. *Bulletin of Entomological Research* **97**: 1-7.

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ABBREVIATIONS AND SYMBOLS

%	percent
µl	microliter
µM	micro molar
°F	degrees fahrenheit
♀	female
♂	male
bp	base pair
cDNA	cloned deoxyribonucleic acid
cm	centimetre
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
EDTA	ethylene diamine tetra acid (disodium salt)
et al.	and others
g	relative centrifugal force
g/100ml	grams per 100 millilitres
g/l	grams per litre
h	hour (s)
HCl	hydrochloric acid
kb	kilo base
KCl	potassium chloride
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre
mM	millimole
mRNA	messenger RNA

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NaAc	sodium acetate
NaCl	sodium chloride
ng	nanogram
nm	nanomole
ºC	degrees celsius
OD	optical density
p	probability level
PCR	polymerase chain reaction
pmol	picomole
rDNA	ribosomal DNA
RNA	ribonucleic acid
rpm	revolutions per minute
s.e.	standard error
Tris	Tris Hydroxymethylaminoethane
UV	ultra violet
V	volts
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organization
χ^2	chi-square

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Malaria is the world's most important vector borne disease and it is widespread throughout the tropics and sub-tropical regions of the world (WHO, 2005). It is endemic in 105 countries and is responsible for over 300 to 500 million clinical cases and at least one million deaths annually (WHO, 1999; Breman, 2001). More than 90% of malaria related deaths occur in Africa and most of these occur in children younger than five years and amongst pregnant women (Philips, 2001; WHO, 2005). Malaria also poses a risk to travellers and immigrants, with imported cases increasing in non-endemic areas (WHO, 2005).

Malaria is caused by infection with a protozoan parasite of the genus *Plasmodium* transmitted through bites from infected female mosquitoes of the genus *Anopheles* (Collins & Paskewitz, 1995). There are over 150 known species of *Plasmodium* and only four of them; *Plasmodium falciparum*, *P. vivax*. *P. ovale* and *P. malariae* infect humans (Collins & Paskewitz, 1995). *Plasmodium falciparum* accounts for the majority of infections and is the most severe (Breman, 2001; WHO, 2005) and is life-threatening in non-immune individuals (Collins & Paskewitz, 1995). *Plasmodium*

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malariae and *P. ovale* infections cause little morbidity and almost no mortality while *P. vivax* infections are more severe and debilitating but are usually self-limiting in healthy individuals (Collins & Paskewitz, 1995). The principal vectors of malaria in Africa are *Anopheles gambiae* s.s. Giles, *An. arabiensis* Patton and *An. funestus* Giles (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987).

Frequent and resurgent malaria epidemics in Africa are attributed to a number of factors including: funding and service delivery, political instability, poverty, insecticide resistance, and the presence of extremely efficient mosquito vectors (Coetzee, 2006). Other factors include war, mass migration of infected people to unaffected areas, differences in the biology of malaria vectors (which preclude the development of universally applicable strategies to malaria control) and finally, the cost of available malaria control often exceeds the financial and public health resources available in malarious countries (Collins & Paskewitz, 1995). The spread of drug-resistant strains of parasites along with the non-availability of effective vaccines has made chemotherapy less effective (Phillips, 2001; WHO, 2004; Davies *et al.*, 2007). As a result, drug combination therapy is now recommended for malaria treatment in areas where resistance to one drug is highly prevalent (WHO, 2001a). In addition to chemotherapy, vector control is one of the most effective ways of preventing malaria transmission (WHO, 2005).

1.2 Malaria vectors

Anopheles species have a worldwide distribution, occurring in both tropical and temperate regions (Service, 1996). There are almost 500 known species of *Anopheles* and only about 20% of these transmit malaria, based on the essential requirements that vectors must be anthropophilic and suscpetible to *Plasmodium* infection (Collins & Paskewitz, 1995). The major vectors of malaria in sub-Saharan Africa are found within the *An. funestus* group and the *An. gambiae* complex (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987; Collins & Paskewitz, 1995).

1.2.1 *Anopheles gambiae* complex

The *An. gambiae* complex is a group of morphologically indistinguishable yet genetically distinct species that differ in their behaviour and vectorial capacity (Gillies & Coetzee, 1987; Hunt *et al.*, 1998). The species within the complex are *Anopheles gambiae* Giles, *An. arabiensis* Patton, *An. quadriannulatus* Theobald species A and B, *An. merus* Dönitz, *An. melas* Theobald and *An. bwambae* White. *Anopheles gambiae* s.s. and *An. arabiensis* are major vectors; *An. bwambae, An. melas* and *An. merus* are minor malaria vectors and *An. quadriannulatus* is a non-vector (White, 1974; Coetzee *et al.*, 2000). *Anopheles gambiae* s.s. is extremely anthropophilic throughout its distribution; *An. arabiensis* is strongly anthropophilic in many parts of its distribution depending on host availability; *An. melas* and *An.*

merus show intermediate anthropophily but are mainly zoophilic and *An. quadriannulatus* is zoophilic (Gillies & Coetzee, 1987).

1.2.2 *Anopheles funestus* Giles

Anopheles funestus is widespread in the Afrotropical Region. It is a group of nine species that are morphologically very similar in the adult stage (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987; Coetzee & Fontenille, 2004). The nine species are *An. aruni* Sobti, *An. brucei* Service, *An. confusus* Evans & Leeson, *An. funestus, An. fuscivenosus* Leeson, *An. leesoni* Evans, *An. parensis* Gillies, *An. rivulorum* Leeson and *An. vaneedeni* Gillies & Coetzee (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987). *Anopheles confusus, An. leesoni, An. rivulorum* and *An. brucei* can be distinguished at the larval stage, *An. fuscivenosus* is known only from the adult stage, while those of the *An. funestus* subgroup (*An. funestus, An. parensis, An. aruni,* and *An. vaneedeni*) can be identified only by minor morphological differences between the adults (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987).

The biology and vectorial capacity of the members of the *An. funestus* complex are quite different (Coetzee & Fontenille, 2004). *Anopheles funestus* is the most anthropophilic and has the widest distribution of the *An. funestus* group (Gillies & De Meillon, 1968). The other members of the group are predominantly zoophilic (Gillies & Coetzee, 1987; Coetzee & Fontenille, 2004). *Anopheles funestus* has been found to attack humans even in the presence of abundant alternative hosts such as sheep and

cattle (Gillies & De Meillon, 1968). *Anopheles funestus* is highly endophilic and feeds mainly during the second half of the night when most people are indoors (Gillies & De Meillon, 1968). Its endophilic nature has made it one of the *Anopheles* species most vulnerable to control using residual insecticides (Gillies & De Meillon, 1968; Coetzee, 2006).

Anopheles funestus breeds preferentially in semi-permanent and permanent water pools and is often associated with rice fields (Gillies & De Meillon, 1968). Its larvae are very difficult to find at low densities due to their tendency to stay submerged for long periods (Gillies & De Meillon, 1968). Fluctuations in populations of adult *An. funestus* follow changes in the level of the water table and in the savannah zones with one period of rainfall per year; numbers start to rise in the middle of the rainy season and peak in the early part of the dry season (Gillies & De Meillon, 1968).

Anopheles funestus females may delay oviposition for four to five days (Hocking & MacInnes, 1948). Normally, two to three days elapse between the taking of blood meals and oviposition (Gillies & De Meillon, 1968). A three-day cycle for the ovarian development has been reported in the highland areas of east Africa and in Cameroon; however, a two-day cycle has been reported to be general throughout Africa (see Gillies & De Meillon, 1968). The length of the first gonotrophic cycle has been reported to be four to five days and two to three days for later cycles in *An. funestus* females in Burkina Faso (see Gillies & Coetzee, 1987).

The interval recorded between oviposition and taking of a subsequent blood meal may vary seasonally (Gillies & De Meillon, 1968). A three-day interval between feeds throughout the year has been reported in Tanzania and in Burkina Faso (Gillies & De Meillon, 1968). Autogeny (ability to lay eggs without taking a blood meal) is unknown in *An. funestus* (Gillies & De Meillon, 1968). In the great majority of newly emerged females, a single blood meal does not suffice for the maturation of eggs (Gillies & De Meillon, 1968). The number of eggs laid by *An. funestus* females varies slightly with age and is mainly correlated with the size of the individual insect (Gillies & De Meillon, 1968).

Male swarms of *An. funestus* are elusive unlike those of other species such as *An. gambiae* (Gillies & De Meillon, 1968). *Anopheles funestus* have been found swarming at dusk 1 - 2 feet above the floor of a veranda house 1 km from Nyanza, Lake Victoria in Kenya (see Gillies & De Meillon, 1968). Charlwood *et al.* (2003) observed swarms of male *An. funestus* within sandy clearings surrounding houses in Mozambique and swarming was co-incident with males leaving houses at sunset. These swarms occurred 2 – 4 m off the ground, occupied a similar volume, and appeared to consist of a similar number of insects.

Anopheles funestus is known to be eurygamic (requiring large volumes of space to successfully mate) and therefore refractory to colonization (Gillies & De Meillon, 1968). Service & Oguamah (1958) succeeded in maintaining a colony in Lagos,

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Nigeria for only few months. However, recently Hunt *et al*. (2005) has successfully maintained colonies of *An. funestus* in South Africa since 2001.

Anopheles funestus is a very efficient vector of human *Plasmodium*, all the other members are believed to be non-vectors (Gillies & De Meillon, 1968). However, *An. rivulorum* has been found to be a minor vector in Tanzania (Wilkes *et al.*, 1996). Wilkes *et al.* (1996) reported the presence of malaria sporozoites in the salivary glands of five out of the 1022 (0.5%) *An. rivulorum* examined from four villages close to the town of Muheza in Tanzania. De Meillon *et al*. (1977) reported that *An. vaneedeni* fed on gametocyte carriers showed full susceptibility to *falciparum* malaria. However, infected mosquitoes have never been found in the field (Gillies & Coetzee, 1987). Mouatcho *et al.* (2007) found that 13.4% (n=149) of *An. parensis* from KwaZulu-Natal, South Africa assayed using the Enzyme-Linked ImmunoSorbent Assay (ELISA) method, tested positive for *P. falciparum.* However, these were later found to be false positives as none of the ELISA positive samples tested positive on re-examination using a polymerase chain reaction (PCR) based method.

Infection rates of *P. falciparum* recorded for *An. funestus* are usually lower than in *An. gambiae* and often fall in the 2 - 5% range (Gillies & De Meillon, 1968). In some places high sporozoite rates have been reported for *An. funestus*, for example a sporozoite rate of up to 50% (n=56) has been recorded in a village in Burkina Faso (Costantini *et al*., 1999). Sporozoite rates reported for *An. funestus* include 0.5%

(n=213) in Okyereko, Ghana (Okoye *et al.*, 2005); 2.42% (n=2181) in Matola, Mozambique (Mendis *et al.*, 2000); 3.8% (n=53) in Ndiop, Senegal (Fontenille *et al.*, 1997); 4% (n=372) in Bioko, Equatorial Guinea (Sharp *et al.*, 2007a) and 5.4% (n=74) in KwaZulu-Natal, South Africa (Hargreaves *et al.*, 2000). Sporozoite rates of 3.5% to 10.8% (Shiff *et al.*, 1995), 6.05% (Temu *et al.*, 1998) and 11% (Temu *et al.*, 2007) have been recorded in coastal Tanzania. *Anopheles funestus* also plays an important part in the transmission of bancroftian filariasis (Gillies & De Meillon, 1968).

1.3 Malaria control

There are two approaches used in malaria control: chemotherapy and preventing contact between humans and vectors using interventions such as insecticides, bednets, environmental management and biological control (Collins & Paskewitz, 1995).

1.3.1 Vector control

Vector control is one of the most effective measures of preventing malaria transmission (WHO, 2005). Mosquito vector control can be directed either against the adult or against the aquatic stages (WHO, 2005). Generally, there is no simple, universally applicable form of vector control because of the wide variety of ecological requirements and behavioural characteristics employed by malaria vectors (Collins & Paskewitz, 1995, Phillips, 2001; Walker & Lynch, 2007).

Considerable efforts have been devoted to biological control because of insecticide resistance and the environmental hazards posed by some insecticides (Collins & Paskewitz, 1995). However, biological control is less effective because larval sites have to be definable and limited in extent for larval control to be viable (Curtis, 1996). The use of pathogens and parasites, including several viruses (e.g. cytoplasmic polyhedrosis, baculovirus); the protozoan *Nosema algerae*; the nematode *Romanomermis culcivorax*; and fungi belonging to the genera *Coelomomyces*, *Culicinomyces, Lagenidium* and *Metarhizium* have shown little practical applicability as biological control agents for mosquito control (Collins & Paskewitz, 1995). However, new advances in developing entomopathogenic fungi for vector control are promising (Blanford *et al.*, 2005; Scholte *et al.*, 2005).

The use of naturally occurring bacteria such as *Bacillus thuringiensis* and *B. sphaericus* has received less attention because of (a) the rapid sedimentation of the spores of the bacilli which is a problem since *Anopheles* larvae are surface feeders; (b) the sensitivity of the spores to UV light; and (c) the killing rate of the spores is lower that that of chemical insecticides (Philips, 2001). However, very effective commercial formulations of *Bacillus thuringiensis israelensis* (Bti) are available for larval control and have been used in large-scale larviciding programmes such as The Gambia (Majambere *et al.*, 2007). Fillinger & Lindsay (2006) found that larviciding with *B. sphaericus* reduced Anopheles larval density by 95% and human exposure to bites from adults by 92% in rural western Kenya.

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The use of various fish species such as *Gambusia*, *Aplocheilus*, *Zacco*, *Oreochromis* and *Poecilia* for mosquito control has been successful in a number of cases (Wickramasinghe & Costa, 1986). For instance field experiments carried out in a Sri Lankan stream stocked with the fish *Aplocheilus dayi* showed a larval reduction rate of 71.1%, 86.6% and 81.5% respectively in three sectors of the stream within one week (Wickramasinghe & Costa, 1986).

Insecticide application (through indoor residual spraying (IRS) and use of insecticide treated nets (ITNs)) is one of the most important components in the global control of malaria vectors (McCarroll & Hemingway, 2002; WHO, 2005). Insecticide treated nets have been shown to reduce the burden of malaria in pregnant women and young children (WHO, 2005). Indoor residual spraying has been useful in reducing mosquito populations for many years and has played a major role in the elimination of malaria various places such as southern Europe and the Mediterranean (Collins & Paskewitz, 1995; WHO, 2005). However, continuous application of insecticide(s) have not been sustainable largely because of the insecticide resistance that has developed in mosquito vectors as well as the toxicity of some insecticide groups (Hargreaves *et al*., 2000; Brooke *et al*., 2001; Hemingway *et al*., 2002; Yawson *et al*., 2004).

1.3.1 Malaria vector control in South Africa

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Malaria is a serious health problem in South Africa and affects about 4.8 million people (10% of the population) (Maharaj *et al*., 2005). The areas affected by malaria in South Africa are only at its very fringes: the three northeastern provinces of KwaZulu-Natal, Mpumalanga and Limpopo. In South Africa, malaria is categorised as seasonal and unstable (Maharaj *et al*., 2005). The major malaria vectors are *An. funestus* and *An. arabiensis* while *P. falciparum* accounts for the majority of malaria-related morbidity and mortality (De Meillon *et al*., 1977; De Meillon, 1986; Maharaj *et al*., 2005).

Historically, anti-larval measures using oil and Paris green were introduced in South Africa in 1932 and were the major means of control until 1946 (Sharp *et al.*, 1988). The first major field trials using a kerosene-pyrethrum mixture as an indoor spray against adult malaria mosquitoes were carried out in 1932 – 1933 in former Natal (KwaZulu-Natal) (De Meillon, 1986). Use of pyrethrum was discontinued in 1946 and replaced by DDT (Dichlorodiphenyltrichloroethane), which was used as both an adulticide and larvicide (Sharp *et al*., 1988). Due to the sustained use of DDT, the predominantly indoor resting mosquito populations of *An. gambiae* s.s. and *An. funestus* s.s. were reduced to undetectable levels in South Africa (De Meillon, 1986) leaving only *An. arabiensis* as the local vector. However, due to the adverse environmental effects of DDT, its use as a larvicide was discontinued in the early 1960s and it was prohibited for agricultural use in 1976 (see Maharaj *et al*., 2005).

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The use of DDT for malaria control in South Africa was phased out in 1996 due to several reasons. These include the following: mounting pressure on the government to protect the environment; resistance to the house-spraying program by local residents within malaria risk areas due to its effect on the biting behaviour of DDT resistant bedbugs; the deposit of a white residue on sprayed surfaces and the discovery of an alternative environmentally friendly insecticide, deltamethrin (Maharaj *et al*., 2005).

After the introduction of pyrethroid insecticides in 1996, there was a marked increase in malaria cases (Figure 1.1) (Coetzee, 2006; Maharaj *et al*., 2005). This was attributed to the advent of drug resistance, high rainfall, the immigration of refugees from neighbouring countries (many of whom carried malaria parasites) and weakening of the malaria control programme (Coetzee, 2006). Entomological surveys conducted in 1999 revealed the presence of *An. funestus* in pyrethroid-sprayed houses and subsequent studies showed that this species was responsible for the malaria transmission with a sporozoite rate of 5.4% (Hargreaves *et al*., 2000). Bioassay studies revealed that these mosquitoes were resistant to pyrethroids (permethrin, deltamethrin and lambda-cyhalothrin) but were still susceptible to DDT (Hargreaves *et al.*, 2000). Owing to the habit of *An. funestus* to breed in permanent water bodies rather than in temporary puddles, it is associated with year-round transmission. This resulted in the tremendous increase of winter malaria observed during the period 1996 - 2000 in South Africa. Further studies showed cross-resistance to the carbamate propoxur, and implicated metabolic detoxification as the mode of

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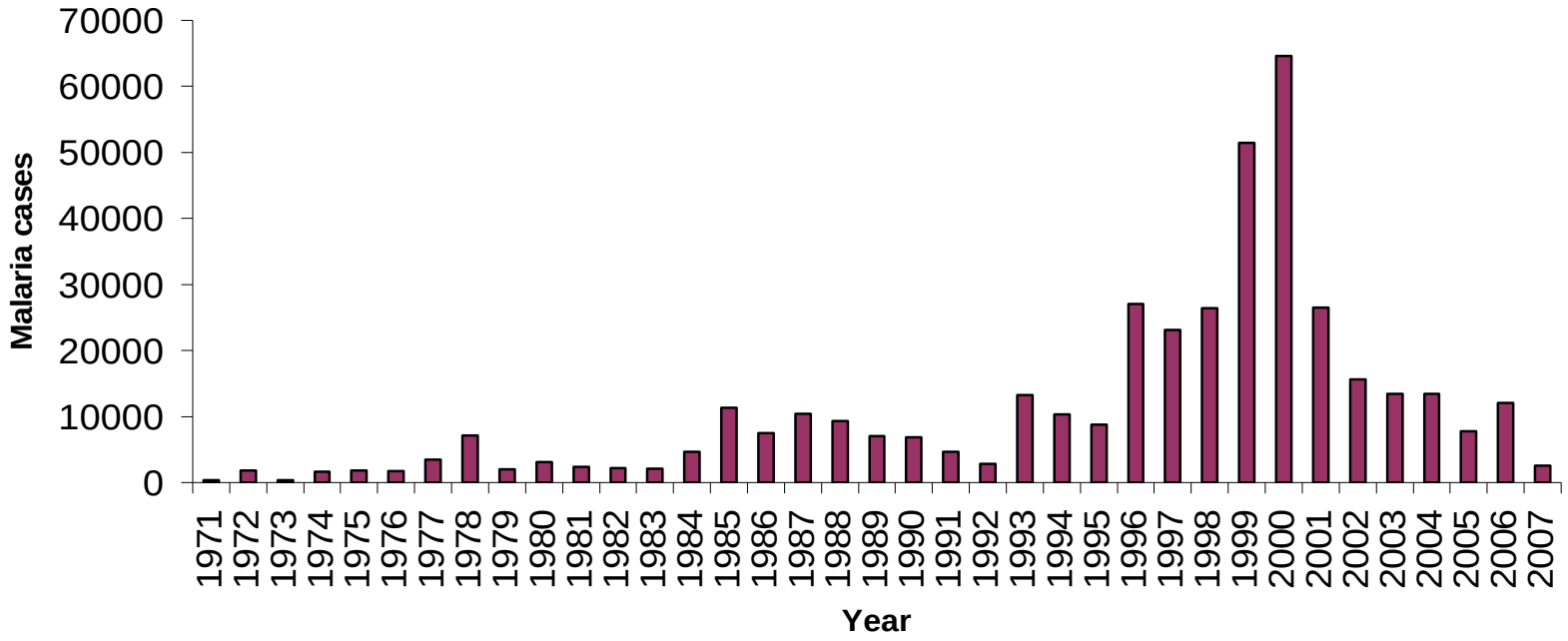
resistance (Brooke *et al.*, 2001). Biochemical analysis and synergist assays identified monooxygenases as the primary mode of detoxification (Brooke *et al.*, 2001). More surveys in the Mamfene region of northern KwaZulu-Natal have identified metabolically mediated DDT resistance in south African *An. arabiensis* (Hargreaves *et al.*, 2003).

Figure 1.1: Malaria cases in South Africa from 1971 to 2007 (Department of Health, Unpublished data). 2007 data represents data for only January to May 2007.



Following the detection of pyrethroid resistance in *An. funestus*, DDT was reintroduced as the best alternative (Maharaj *et al*., 2005). Since 2001, indoor residual spraying using DDT and pyrethroids has been conducted in October each year in Mpumalanga, Limpopo and KwaZulu-Natal provinces (Sharp *et al*., 2007b). Pyrethroids are currently being used in western-type structures (cement-plastered or painted) while DDT is used in traditional structures (mud, reed or wood) (Coetzee, 2006; Sharp *et al*., 2007b).

1.4 Insecticide resistance

Insecticide resistance is defined by the World Health Organisation as "the development of an ability in some individuals of a given organism to tolerate doses of a toxicant which would prove lethal to a majority of individuals in a normal population of the same species" (from Liu *et al*., 2006). The development of resistance is influenced by many factors (Georghiou & Taylor, 1986). These include (a) genetic factors including the level of resistance conferred by the resistance allele(s), mutation and frequency of the resistant gene(s) and relative dominance of the characters; (b) biological factors including the fitness of the heterozygous and homozygous resistant phenotypes and initial population size; (c) reproductive factors including rate of increase and fluctuations in population size; and (d) operational factors including previous selection with other insecticides, mode of insecticide application, proportion of population exposed to selective doses, dosage of insecticide taken up by exposed insects and the life stage of the mosquito selected (Georghiou &

Taylor, 1986; Georghiou & Taylor, 1986; Tabashnik, 1986; WHO (online) (http://www.emro.who.int/rbm/Publications/InsecticideResistance.pdf.).

Since the 1940s and 1950s, resistance has appeared in most major insect vectors from every genus except *Glossina* (WHO, 1992). More than 100 mosquito species including 56 species of anopheline and 39 species of culicine mosquitoes are known to have developed resistance to one or more insecticides (WHO, 1992). Pyrethroid resistance has been detected in various *Anopheles* species including *An. gambiae* (Chandre *et al.*, 1999; Yawson *et al.*, 2004; Casimiro *et al.*, 2006a; Corbel *et al.*, 2007; N'Guessan *et al.*, 2007), *An. albimanus* (Brogdon & Barber, 1990) and *An. arabiensis* (Casimiro *et al.*, 2006a). Organophosphate or malathion resistance has been recorded in all the major *Culex* and *Anopheles* vectors species including *An. arabiensis* (Hemingway, 1983), *An. culicifacies* (Herath *et al.*, 1987), *An. stephensi* (Hemingway, 1982), *An. albimanus* (Ariaratnam & Georghiou, 1974) and *An. sacharovi* (Hemingway *et al.*, 1985). Carbamate resistance has been detected in *An. gambiae* (N'Guessan *et al.*, 2003), *An. sacharovi* and *An. albimanus* (Hemingway *et al.*, 1992). Reports of insecticide resistance in *An. funestus* include resistance to dieldrin in Benin, Cameroon, Ghana, Kenya, Mali and Nigeria (Brown, 1986); and to DDT, malathion and fenitrothion in Mali (Toure, 1984). Other reports of resistance in *An. funestus* include resistance to DDT and bendiocarb in Obuasi, Ghana (Coetzee *et al.*, 2006); deltamethrin in KwaZulu-Natal, South Africa and in southern Mozambique (Hargreaves *et al.*, 2000; Brooke *et al.*, 2001) as well as resistance to

pyrethroid and carbamates (propoxur and bendiocarb) in Mozambique (Casimiro *et al.*, 2006b).

1.5 Insecticide modes of action and mechanisms of resistance

Insecticides generally target the nervous system of the insect. Organophosphate (e.g. chlorpyrifos, diazinon, fenitrothion, fenthion, malathion and temephos) and carbamate (e.g. propoxur, bendiocarb, carbaryl) insecticides are cholinesterase inhibitors. Cyclodienes (e.g. dieldrin) insecticides affect the chloride channel by inhibiting the gamma aminobutyric acid (GABA) receptor. Pyrethroids (e.g. permethrin, deltamethrin, cypermethrin) and DDT act on the sodium channel preventing these channels from closing, resulting in continual nerve impulse transmission, tremors, and eventually death (Bloomquist, 1996).

The mechanism of insecticide resistance adopted by an organism depends on the prevailing pressure and on the mode of action of the insecticide in use. Insects become resistant to insecticides mainly through increased metabolic detoxification and decreased target site sensitivity (Mourya *et al.*, 1993; Liu *et al.*, 2006). In the case of metabolic detoxification, the insecticide is prevented from reaching its site of action. On the other hand, decreased target site sensitivity reduces the rate at which the insecticide binds to its target site (Brogdon & McAllister, 1998; Pasteur & Raymond, 1996; Hemingway, 2000). All these mechanisms are non-specific and may

confer cross-resistance to other structurally related insecticides. These mechanisms are detailed below.

1.5.1 Target Site Insensitivity

Resistance due to modification of neural target sites has been identified for acetylcholinesterase, gamma aminobutyric acid (GABA)-gated chloride channel and the voltage-sensitive sodium channel. These are detailed below.

1.5.1.1 Altered acetylcholinesterase

The mode of action of organophosphates and carbamate insecticides involves alterations in acetylcholinesterase (AChE) (Hemingway, 1989; Brogdon & McAllister, 1998; Hemingway & Ranson, 2000). This resistance mechanism is due to a change in the AChE, reducing its affinity for the insecticides and, in most cases, to some extent for its normal substrate acetylcholine (Hemingway, 1989). Altered AChE has been found to confer resistance to propoxur and three organophosphate insecticides (temephos, chlorpyrifos and malathion) in *C. pipiens* from Portugal (Bourguet *et al*., 1996). The presence of altered AChE has also been detected in an organophosphate (ethyl parathion) and carbamate (propoxur) resistant strain of *An. albimanus* (Hemingway & Georghiou, 1983); carbamate resistant field populations of *An. albimanus* from southern Mexico (Penilla *et al*., 1998); field populations of *An. nigerrimus*, *C. tritaenorhynchus* and *C. gelidus* from Sri Lanka (Hemingway &

Smith, 1986; Hemingway *et al.*, 1986; Karunaratne & Hemingway, 2000); and in field populations of *An. sacharovi* from Cukurova plain of Adana province, Turkey (Hemingway *et al.*, 1992). The presence of an altered AChE gene has also been detected at a low frequency in pyrethroid resistant populations of *An. gambiae*, *An. funestus* and *An. arabiensis* from Mozambique (Casimiro *et al.*, 2006a; Casimiro *et al.*, 2006b).

1.5.1.2 Gamma aminobutyric acid (GABA) receptors

The gamma aminobutyric acid (GABA) receptor is a chloride-ion channel in the insect's central nervous system and neuromuscular junctions (Hemingway & Ranson, 2000). Mutations of the GABA receptor are implicated as a site of action for ivermectins and cyclodienes (Hemingway & Ranson, 2000). A single mutation of alanine302 with a serine in the M2 transmembrane domain of the GABA gene is associated with dieldrin resistance of *Drosophila melanogaster* (Ffrench-Constant *et al.*, 1993). This mutation has been found to be present in dieldrin resistant strains of the yellow fever mosquito *Aedes aegypti*, the house fly *Musca domestica*, the red flour beetle *Tribolium castaneum*, the coffee pod borer *Hypothenemus hampei* and the American cockroach *Periplaneta americana* (Ffrench-Constant *et al*., 1993). A mutation of alanine296 to glycine has been associated with dieldrin resistance in *An. gambiae* (Du *et al.*, 2005; Brooke *et al.*, 2006). Another mutation of alanine to serine at the same codon has also been associated with dieldrin resistance in a laboratory strain of *An. arabiensis* (Du *et al.*, 2005).

1.5.1.3 Sodium ion channels (Knock down resistance (*kdr*))

Voltage-gated sodium channels are the target for both pyrethroid insecticides and DDT, by the insecticide altering the function of the sodium channels in nerve membranes (Soderlund & Bloomquist, 1989; Soderlund & Knipple, 2003). Reduced sensitivity of the sodium channel to DDT and pyrethroids is expressed as "knockdown resistance" (*kdr*) (Soderlund & Knipple, 2003).

Knockdown resistance was first described in the house fly, *Musca domestica* with substitution of leucine (TTA) to phenylalanine (TTT) resulting from a single nucleotide polymorphism (SNP), termed the *kdr* mutation, in domain II, segment 6 of the sodium channel gene (Williamson *et al*., 1996; Soderlund & Knipple, 2003). This is the most common *kdr* mutation (Davies *et al*., 2007). Two alternative substitutions at this position also confer resistance to DDT and/or pyrethroids: a leucine to histidine substitution is associated with pyrethroid resistance in the tobacco budworm *Heliothis virescens* (Park & Taylor, 1997); and a leucine to serine substitution confers DDT resistance and low levels of permethrin resistance in a strain of *C. pipiens* from China (Martinez-Torres *et al*., 1999; Jamroz *et al*., 1998), east African *An. gambiae* (Ranson *et al*., 2000a), and *An. sacharovi* (Lüleyap *et al*., 2002). Four novel mutations of leucine to tryptophan, isoleucine to methionine, glycine to valine, and valine to glycine, have been identified in the domain II of the sodium channel gene of

pyrethroid resistant field population of *Ae. aegypti*, in which the *kdr* mutation is absent (Brengues *et al*., 2003).

The *kdr* mutation has been recorded in pyrethroid resistant populations of *An. gambiae* in several African countries. These include Burkina Faso (Martinez-Torres *et al*., 1998; Weill *et al.*, 2000; Diabate *et al*., 2004a), Cameroon (Etang *et al*., 2006), Cote d'lvoire (Chandre *et al.*, 1999; Weill *et al.*, 2000) and Equatorial Guinea (Reimer *et al*., 2005; Sharp *et al*., 2007a). Others include Benin (Weill *et al.*, 2000), Central Africa (Weill *et al.*, 2000), Gabon (Pinto *et al*., 2006), Ghana (Yawson *et al*., 2004), Kenya (Ranson *et al*., 2000a), Mali (Fanello *et al*., 2003), Nigeria (Awolola *et al*., 2002), Senegal (Weill *et al.*, 2000) and Uganda (Verhaeghen, *et al*., 2006). The *kdr* mutation has also been detected in *An. stephensi* (Enayati *et al.*, 2003), *An. arabiensis* (Diabate *et al*., 2004b; Verhaeghen, *et al*., 2006; Matambo *et al.*, 2007), *An. sacharovi* (Lüleyap *et al*., 2002), *C. pipiens* (Martinez-Torres *et al.*, 1999; McAbee *et al.*, 2004), and *C. quinquefasciatus* (Xu *et al*., 2005).

Another mutation of methionine to threonine known as the super-*kdr* mutation occurs between the S4 and S5 segments of domain II and results in a much higher resistance than *kdr* in house flies (Miyazaki *et al*., 1996; Williamson *et al*., 1996); horn fly (Guerrero *et al.*, 1997); diamondback moth (Schuler *et al*., 1998); head louse (Lee *et al*., 2000) and tobacco whitefly (Morin *et al*., 2002). These super-*kdr* mutations are usually found in combination with the *kdr* mutation (Soderlund & Knipple, 2003). More than twenty different sodium channel mutations has been identified in different

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resistant arthropods and all those mutations so far identified in resistant mosquitoes are in domain II of the sodium channel gene (Soderlund & Knipple, 2003; Hemingway *et al.*, 2004; Liu *et al.*, 2006).

1.5.2 Increased metabolic detoxification

Increased metabolic detoxification is one of the most common mechanisms of insecticide resistance (Hemingway & Karunaratne, 1998; Hemingway, 2000). Three enzyme classes are involved in insecticide detoxification: the monooxygenases (cytochrome P450s), esterases and glutathione S-transferases (Pasteur & Raymond, 1996; Brogdon & McAllister, 1998; Hemingway & Karunaratne, 1998; Hemingway, 2000; Liu *et al*., 2006).

1.5.2.1 Monooxygenases

The monooxygenases are a complex of detoxifying enzymes found in most aerobic organisms including insects and are critical in the regulation of endogenous compounds such as drugs, insecticides and plant toxins (Scott, 1999; Hemingway & Ranson, 2000). Cytochrome P450 monooxygenase mediated detoxification is a very important resistance mechanism because they metabolize virtually all insecticides (Scott, 1999; Hemingway & Ranson, 2000).

The spectrum of insecticide resistance conferred by monooxygenases includes pyrethroids, organophosphates and to lesser extent carbamates (Pasteur & Raymond, 1996; Hemingway & Ranson, 2000). Monooxygenase mediated resistance has been found in *An. stephensi*, *An. subpictus* and *An. gambiae* (Hemingway *et al.,* 1991; Vulule *et al.,* 1994; Brogdon *et al.,* 1997). Monooxygenases have also been reported to be responsible for pyrethroid resistance in *An. funestus* from southern Africa and Mozambique (Hargreaves *et al.*, 2000; Brooke *et al.*, 2001; Casimiro *et al.*, 2006a), *C. quinquefasciatus* (Chandre *et al.,* 1998; Kasai *et al.,* 1998), *An. gambiae* and *An. arabiensis* from Mozambique (Casimiro *et al.,* 2006a). Monooxygenases have been found to confer permethrin resistance and possibly cross-resistance to other pyrethroids in a strain of *An. gambiae* from Kenya (Vulule *et al.,* 1999), and carbamate resistance in a population of *Ae. aegypti* from Venezuela (Mazzarri & Georghiou, 1995).

1.5.2.2 Esterases

Esterases produce a broad range of insecticide resistance through sequestration of the insecticide rather than metabolizing the insecticide (Hemingway & Karunaratne, 1998; Hemingway, 1999; Hemingway & Ranson, 2000). They can also provide a narrow range of insecticide resistance through metabolism of a few insecticides with an ester bond (Hemingway & Karunaratne, 1998). In mosquitoes, esterase-based resistance mechanisms are either through (a) the esterase is modified so that they metabolize insecticides more efficiently; or (b) the esterase is elevated, primarily

through gene amplification (Hemingway, 1999). The most common organophosphate resistance mechanism in more than 90% of *C. quinquefasciatus* strains is the co amplification of two esterases, *Estα2*[1] (or esterase A) and *Estβ2*[1] (or esterase B) genes (Vaughan & Hemingway, 1995; Hemingway, 1999). Esterases detoxify organophosphates and carbamates and are important to a lesser extent in resistance to pyrethroids (Pasteur & Raymond, 1996; Hemingway & Ranson, 2000).

Esterase mediated detoxification has been described in field populations of *An. albimanus* from southern Mexico (Penilla *et al*., 1998), pyrethroid resistant populations of *An. gambiae* and *An. arabiensis* from Mozambique (Casimiro *et al*., 2006a) and a temephos resistant strain of *C. quinquefasciatus* from Colombia (De Silva & Hemingway, 2002). Resistance to temephos and chlorpyrifos have been associated with esterases in *Ae. aegypti* (Mazzarri & Georghiou, 1995). The same enzyme is associated with malathion resistance in *C. quinquefasciatus* from Cuba (Coto *et al*., 2000) and in *C. quinquefasciatus* and *C. tritaeniorhynchus* from Sri Lanka (Karunaratne & Hemingway, 2001). Esterases have also been found to confer resistance to permethrin in *An. gambiae* from Kenya (Vulule *et al*., 1999).

1.5.2.3 Glutathione S-Transferases

Glutathione S-transferases (GSTs) confer resistance by conjugating reduced glutathione (GSH) to a large range of xenobiotics aiding in their detoxification and excretion (Hemingway, 1999; Hemingway, 2000; Hemingway & Ranson, 2000).

Some GSTs use organophosphates in the conjugation reaction while others detoxify DDT to the non-toxic metabolite DDE using GST as a co-factor (Hemingway, 1999). GSTs play a role in the detoxification of organophosphates and DDT (Pasteur & Raymond, 1996; Hemingway, 1999). Elevated GST levels are found in many resistant insect species (Ahammad-Sahib *et al*., 1994; Feyereisen, 1999). They have been implicated in most reports of DDT resistance in *Anopheles* species (Prapanthadara *et al*., 1995). Examples include DDT resistance in *An. arabiensis* (Hargreaves *et al.,* 2003), *An. gambiae* (Prapanthadara *et al*., 1996), *An. atroparvus* and *An. sacharovi* (Hemingway *et al*., 1992) and *An. albimanus* (Penilla *et al*., 1998). There are also cases of cross-resistance between DDT and some organophosphates (Hemingway, 1999). For example, in *An. subpictus* from Sri Lanka, GST-based DDT resistance often acts as a secondary resistance mechanism for fenitrothion with a monooxygenase or esterase-based resistance mechanism (Hemingway *et al*., 1991).

1.6 The genetic basis of resistance

The characteristic of resistance is inherited either as a single genetic factor (monogenic) or as multiple genetic factors (polygenic) (Brown, 1986; Uyenoyama, 1986). Different classes of theoretical models are applicable to either monogenic or polygenic mode of inheritance (Uyenoyama, 1986; Roush & McKenzie, 1987). When there are numerous genes, cross-resistance associated with each gene is limited. On the other hand, when few genes are involved, then resistant populations would be

expected to show cross-resistance to insecticides to which they have never been exposed (Plapp, 1986).

A resistance allele may be inherited as a recessive, dominant or co-dominant trait (with the progeny of the resistant-susceptible crosses being intermediate) (Brown, 1986). It is important to determine whether resistance is effectively recessive at field dosages, because at low resistance gene frequencies almost all the genes would be heterozygotes and if these are killed by field exposures, the gene will gain very little selective advantage.

Davidson (1956) reported that dieldrin and BHC resistance was monofactorial with incomplete dominance in a strain of *An. gambiae* from northern Nigeria. Davidson (1958) further reported that dieldrin resistance in *An. gambiae* and DDT resistance in *An. sundaicus* was monofactorial. Haridi (1970, 1972) and Ranson *et al.* (2000b) showed that DDT resistance in *An. gambiae* was monofactorial with either complete or incomplete dominance. The inheritance of the *kdr* mutation conferring DDT and pyrethroid resistance has been found to be incompletely recessive in *An. gambiae* (Martinez-Torres *et al.*, 1998; Chandre *et al.*, 2000). DDT resistance in *An. stephensi* has been reported to be inherited in a fully dominant monofactorial manner (Davidson, 1958; Malcolm, 1990). Qutubuddin (1958) reported that a single gene was involved in inheritance of DDT resistance in *Ae. aegypti*. Esterase based malathion resistance in *An. culicifacies* (Herath *et al.*, 1987), *An. stephensi* (Hemingway, 1982)

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and *An. arabiensis* (Hemingway, 1983) is inherited monofactorially as a semi-dominant autosomal trait.

Mebrahtu *et al.* (1997) reported that permethrin resistance in *Ae. aegypti* was inherited as partly recessive in the F1 hybrids when the resistant parent was male and inheritance was partially dominant when the resistant parent was female. Priester & Georghiou (1979) found pyrethroid resistance to be inherited in a co dominant and partially recessive manner in two different strains of *C. quinquefasciatus*. Inheritance of *B. sphaericus* resistance in *C. quinquefasciatus* resistance has been demonstrated to be recessive, monofactorial and autosomal (Oliveira *et al*., 2004).

1.7 Fitness cost of insecticide resistance

Biological fitness is defined as reproductive success or as the proportion of genes that an individual leaves in the gene pool of the population (see Rodcharoen & Mulla, 1997). Resistance of mosquitoes to insecticides is an adaptation to a disadvantageous environment. If the frequencies of resistance genes are low before insecticide selection, it can be inferred that they are disadvantageous and represent a significant reproductive disadvantage in the absence of selection (Crow, 1957). Where resistance alleles confer a significant reproductive disadvantage, natural selection against resistance alleles in the absence of insecticide treatment would assist in limiting the rate of development of resistance in insect populations (Georghiou & Taylor, 1977).

In the absence of selection for resistance genes by insecticides they would be re-generated in the population by mutation.

Fitness costs of insecticide resistance genes can be studied using two general methods (Roush & McKenzie, 1987). The first method involves following changes in the genotypic frequencies of resistance alleles in populations that were not treated with insecticides for several discrete or overlapping generations (Roush & McKenzie, 1987). The second involves comparing fitness components (such as fecundity, developmental time and fertility) between resistant and susceptible individuals with the assumption that the resistance individuals can only have an advantage in the presence of insecticide (Roush & McKenzie, 1987; Bourguet *et al*., 2004).

Numerous studies based on a variety of physiological, behavioural and fecundity measures have shown that resistant strains of arthropods often demonstrate lower fitness compared with their susceptible counterparts (Georghiou & Taylor, 1986). For example, various laboratory studies have shown that resistance strains may be associated with relatively slower larval development, reduced survival rates amongst larvae and adults, reduced fecundity in females and reduced fertility (Rowland, 1991; Wang *et al.,* 1998; Campanhola *et al*., 1991). Reduced fecundity and reduced response to oviposition site has been recorded in γHCH/dieldrin resistant strains of *An. gambiae* and *An. stephensi* (Rowland, 1991). Ferrari & Georghiou (1981) found that a temephos resistant strain of *C. quinquefasciatus* exhibited reduced fecundity and fertility compared with a similar susceptible strain that had been maintained

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without selection. Reduced biotic fitness (longer pre-adult stage developmental time) was found in a chlorpyrifos resistant strain of *C. quinquefasciatus* compared to a standard susceptible strain (Amin & White, 1984). Wang *et al.* (1998) reported that resistance was disadvantageous to the blood feeding, oviposition and development of three organophosphate (dipterex, temephos and chlorpyrifos) resistant strains of *C. pipiens pallens*.

Reduced fecundity, fertility and slower developmental time has also been recorded in a *B. sphaericus* resistant strain of *C. quinquefasciatus* (Rodcharoen & Mulla, 1997; Oliveira *et al.*, 2003); pyrethroid (permethrin and cypermethrin) resistant strain of the tobacco budworm, *Heliothis virescens* (Campanhola *et al*., 1991); *B. thuringiensis* resistant Indianmeal moth, *Plodia interpunctella* (Oppert *et al.*, 2000); endrin resistant strain of the boll weevil, *Anthonomus grandis* (Thomas & Brazzel, 1961) and an azinphosmethyl resistant strain of the Colorado potato beetle, *Leptinotarsa decemlineata* (Argentine *et al.*,1989). Alyokhin & Ferro (1999) reported that a *B. thuringiensis* resistant strain of *L. decemlineata* produced fewer eggs and larvae than the susceptible strain. Carrière *et al.* (2001) also found reduced survival in two *B. thuringiensis* resistant strains of the pink bollworm, *Pectinophora gossypiella* relative to the susceptible strain.

However, there are many cases where differences in relative fitness between resistant and susceptible strains are found to be negligible and some cases where the resistant strain appears to have an advantage (Varzandeh *et al.*, 1954; Heather, 1982; Beeman

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& Nanis, 1986; Arnaud *et al.*, 2002). Emeka-Ejiofor *et al.* (1983) found no significant differences in the survivorship of the pre-adult stages of dieldrin resistant, DDT resistant and susceptible strains and F1 heterozygotes of *An. gambiae*. A shorter incubation period and greater longevity has been found in DDT resistant strains of the German cockroach, *Blatella germanica* than in the susceptible strains (Perkins & Grayson, 1961). Haubruge & Arnaud (2001) found that a malathion specific resistant strain of *T. castaneum* showed an 8 - 23% increase in biotic potential (fecundity and developmental time) relative to the susceptible strain.

Absence of a fitness cost has also been recorded in a dieldrin resistant *An. albimanus* (Gilotra, 1965); a malathion resistant population of the rice weevil, *Sitophilus oryzae* (Heather, 1982); a permethrin resistant strain of *L. decemlineata* (Argentine *et al.*, 1989); a malathion resistant strain of the parasitoid *Anisopteromalus calandrae* (Baker *et al.*, 1998); a DDT resistant (Varzandeh *et al.*, 1954) and a diazinon resistant (Whitehead *et al.*, 1985) strain of the house fly, *M. domestica* and in a malathion resistant red flour beetle, *T. castaneum* (White & Bell, 1988; Arnaud *et al.*, 2002). Roush & Hoy (1981) also found that the vigour and reproductive compatibilities of a carbaryl resistant strain of a predatory mite, *Metaseiulus occidentalis* did not differ significantly from the susceptible strain in the absence of carbaryl treatment.

Georghiou & Taylor (1977) suggested that when selection for resistance was relaxed, mosquitoes would regress to susceptibility because of the disadvantage of resistance genes under natural selection. However, it is difficult to associate fitness

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disadvantages recorded in laboratory conditions to resistance in field populations (Roush & Croft, 1986; Roush & McKenzie, 1987; Oliveira *et al.*, 2003) because resistance and fitness may evolve independently (Heather, 1982). In addition, the immigration of susceptible individuals into resistant populations under field conditions may cause a decline in resistance gene frequencies (Roush & McKenzie, 1987). However, laboratory data on the relative reproductive and survival rates of resistant and susceptible genotypes are useful when considering the influence of resistance alone on biological fitness (Rodcharoen & Mulla, 1997).

1.8 Rationale of the study

The discovery of insecticide resistance in *An. funestus* in the affected provinces of South Africa has prompted an urgent enquiry into the necessity of incorporating insecticide resistance management strategies into formal malaria control policy in the provinces. These resistance management strategies rely on the assumption of reduced fitness in vector populations associated with resistance genes (Bonning & Hemingway, 1991; Raymond *et al.*, 2001) and are based on the principle that resistance genes will tend to be selected out of vector populations in the absence of insecticide selection pressure. Given the direct relationship between insecticide resistance mechanism and relative general fitness, characterisation of the mechanism of resistance is the first step required in order to understand the effect of resistance on fitness. The mechanism of pyrethroid resistance has been described for southern

African *An. funestus* (Brooke *et al.*, 2001) but remains undescribed for other *An. funestus* populations such as that from the Obuasi region of Ghana.

Although, several studies have been conducted on the relative fitness of insecticide resistant strains of various species of mosquitoes to various natural and synthetic insecticides (Ferrari & Georghiou, 1981, Amin & White, 1984, Bonning & Hemingway, 1991, Rowland, 1991; Rodcharoen & Mulla, 1997), there are currently no published studies on fitness related to resistance in *An. funestus*.

1.9 Objectives

In order to exploit the reduced fitness that might be associated with insecticide resistance, it is important to establish the relative fitness of pyrethroid resistant *An. funestus* mosquitoes. This study aimed to determine whether the pyrethroid resistance in *An. funestus* affects certain fitness components when compared to fully susceptible counterparts. Besides elucidating the effects of insecticide resistance on the resistant strains, this study also aimed to establish the mode of inheritance and phenotypic expression of the resistance factor(s) and determine the stability of resistance in the absence of insecticide selection pressure. In addition, the study also aimed to provide information on the sodium channel gene of resistant and susceptible strains of *An. funestus* from southern Africa including comparing them to that of Ghanaian field populations of *An. funestus* as an out group. Further characterisation of DDT and pyrethroid resistance in Ghanaian *An. funestus* led on from this. Understanding these

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aspects is essential for improving resistance monitoring, detection, and management in vector control programs in South Africa, and provides baseline data for similar studies on Ghanaian *An. funestus*.

1.9.1 Specific objectives

Specific objectives were:

1. To establish the relative fitness of insecticide resistant southern African *An. funestus* compared to their fully susceptible counterparts.
2. To investigate the mode of inheritance and phenotypic expression of the pyrethroid resistance factor/s in southern African *An. funestus*.
3. To monitor the stability of resistance expression in a pyrethroid resistant strain of southern African *An. funestus* in the absence of insecticide selection pressure through several generations.
4. To sequence, analyze and compare the domain II region of the sodium channel gene between pyrethroid resistant and susceptible strains of southern African *An. funestus*.
5. To sequence, analyze and compare the domain II region of the sodium channel gene between pyrethroid/DDT resistant and susceptible samples of Ghanaian *An. funestus* as well as to compare them to the southern African *An. funestus*.

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6. To characterize the mechanism/s conferring pyrethroid and DDT resistance in Ghanaian *An. funestus.*

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CHAPTER 2

RELATIVE DEVELOPMENTAL AND REPRODUCTIVE FITNESS ASSOCIATED WITH PYRETHROID RESISTANCE IN *AN. FUNESTUS*

2.1 INTRODUCTION

The decision to revert to using DDT for malaria vector control in South Africa was based on bioassays showing that the pyrethroid resistant mosquitoes were not cross resistant to DDT (Hargreaves *et al*., 2000). This lack of cross resistance was later explained by the identification of monooxygenase mediated pyrethroid resistance in *An. funestus* populations in KwaZulu-Natal and southern Mozambique (Brooke *et al*., 2001). Although DDT has retained its effectiveness against *An. funestus*, sociological considerations and the development of DDT resistance in *An. arabiensis* in KwaZulu-Natal (Hargreaves *et al*., 2003) have indicated that alternatives to DDT will need to be identified. An alternative strategy will need to incorporate the concept of insecticide resistance management.

Resistance management strategies often rely on the assumption of reduced fitness in vector populations associated with resistance genes (Curtis *et al*., 1978; Bonning & Hemingway, 1991; Raymond *et al.*, 2001). Although numerous fitness studies have been carried out on various insecticide resistant insects as detailed in Chapter 1, there

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are currently no published studies on the possible fitness cost associated with insecticide resistance in *An. funestus*. In this chapter, the aim was to determine whether pyrethroid resistance in southern African *An. funestus* affects certain fitness components when compared to their fully susceptible counterparts.

2.2 MATERIALS AND METHODS

2.2.1 Laboratory strains of *An. funestus*

FUMOZ-R: A pyrethroid (permethrin) resistant selected strain originating from southern Mozambique (FUMOZ) (Hunt *et al*., 2005) and kept in colony since July 2001. This strain was selected on 1.5% permethrin papers for a period of 1 h using double the dosage recommended by the WHO protocol for testing adult anopheline susceptibility to insecticides (WHO, 1998) and currently shows 0 - 1% mortality at this dosage.

FANG: A strain originating from southern Angola and kept in colony since January 2003. It is fully susceptible to pyrethroid insecticides. Specifically, adult males and females exposed to 0.75% permethrin for 1 h consistently show 100% mortality 24 h post exposure.

2.2.1.1 Rearing conditions

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All life stages were reared in standard insectary conditions of 25 ± 3°C, 80 ± 10% relative humidity (RH), with a 12-hour day/night cycle and 30 min dusk/dawn transition period. All adults were fed on a 10% sugar solution and larvae were fed on a mixture of finely ground dog biscuits and brewer's yeast (Hunt *et al*., 2005). Adult females were blood-fed three times per week.

2.2.2 Life table experiments

Comparative fitness was assessed using the following parameters: fecundity, fertility, mating success, rate of larval and pupal development and survival, adult survival and sex ratios of adults. Comparisons were made between susceptible (FANG) and resistant (FUMOZ-R).

2.2.2.1 Fecundity and fertility

Mass mating was carried out by placing newly emerged males and females from the susceptible and resistant strains in screened cages. They were left to mate for 10 days and provided with a 10% sucrose solution. This is because the *An. funestus* feeding/egg laying cycle is longer that that of any other species of *Anopheles* and *Aedes aegypti* known from laboratory colonies (R. Hunt pers. comm.). Samples of 30 surviving females from each strain were offered blood meals on the tenth day and then placed individually in glass vials lined with damp filter paper (see Appendix A,

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Figure A.1) to enable them to lay eggs. Isolated females were subsequently blood-fed three times a week and provided with a 10% sucrose solution.

Every 24 h, the glass vials were checked for eggs and all eggs laid were removed and placed in rearing bowls (one bowl per family) containing approximately 200 ml of distilled water for hatching (see Appendix A, Figure A.2). Eggs from individual families were counted. Each family was labelled with a unique female identifying number that indicated mosquito strain, identity number and date of oviposition. The same identifying number was used to identify all the life stages of each family until adult emergence.

Time to first egg production, number of eggs laid per female for each strain, number of eggs that hatched and time from egg laying to hatching were recorded. Fecundity was measured as the mean number of eggs produced per female while the percentage egg hatch was recorded as a measure of fertility.

2.2.2.2 Development time and survivorship

Families of larvae from each of the thirty females from the susceptible and resistant strains were reared in bowls (one bowl per family) containing approximately 200 ml of distilled water (see Appendix A, Figure A.3). The water level was kept constant by adding water when necessary. Larval bowls were large enough to allow for a

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sufficient surface area (34 cm X 27 cm = 918 cm^2) to prevent overcrowding and competition for food. The number of larvae varied between families. Larvae were fed twice a day with the amount provided dependent on how much of the previous meal had been consumed. Larvae in all rearing bowls were reared through to adults in the same insectary under the same conditions in order to minimise environmental variation. Larvae were counted daily until pupation.

The pupae were transferred daily to a plastic 50 ml container and placed in screened cages for adult emergence. The number surviving at each stage was recorded daily. From pupation to adult emergence the number of pupae and adults per family were counted daily until all pupae emerged into adults or died.

The number of adults that emerged from each family was counted and the percentage of adult emergence was calculated on the basis of total number of eggs, larvae and pupae per family. Number of females and males produced per family were counted and the sex ratio was calculated for each family.

The proportion of larvae surviving to the pupal stage, day of first pupation and proportion of pupae surviving to adult stage was recorded. The mean number of days spent in each stage was recorded. This included the mean length of time from egg to larva, larva to pupa, pupa to adult, egg to first adult emergence, egg hatch to first adult emergence and the generation time from adult to adult for each strain (n=30 families per strain).

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2.2.2.3 Egg production

In order to determine the proportion of females that mate and produce eggs, separate samples of 30 randomly selected females were isolated from the resistant (FUMOZ-R) and susceptible (FANG) strains after an initial 10 day mating period in cages of mixed males and females. These were not the same samples as those described in the *fecundity and fertility* and *Development time and survivorship* sections. The separated females were provided with a 10% sucose solution. The selected females were individually placed in glass vials lined with filter paper and offered blood meals three times a week until they eventually died.

Every 24 h, the glass vials were checked for eggs and all eggs laid were removed from the vials and placed in rearing bowls containing approximately 200 ml of distilled water for hatching. The number of females that laid eggs as well as the number of families that hatched was recorded in order to determine the mating success rate of each strain. The percentage of females that laid eggs in which all eggs failed to hatch was considered to be either sterile or unmated whilst those that laid viable eggs were considered to be fertile. During the experiment, a high mortality was noted in the FANG strain adults which might be an indication reduced fitness and the experiment had to be repeated three times in order to confirm the results.

2.2.2.4 Adult longevity

Cohorts of 0 - 1 day old males and females were separated at emergence and 50 of each sex from the susceptible and resistant strains were set up in screened cages and provided with a 10% sucrose solution. There were five replicates for each strain. Females were offered blood meals after 4 days, and were re-fed at 2-day intervals. Every 24 h, dead adults were removed and the number of deaths and sex of dead adults was recorded. The spermathaecae of dead females were dissected under a dissecting microscope for the detection of sperm as an indication of mating success.

2.2.2.5 Effect of insecticide exposure on fecundity and fertility of pyrethroid resistant *An. funestus*

In order to determine the effect of insecticide treatment on the fecundity and fertility of resistant (FUMOZ-R) female *An. funestus*, 0 – 1 day old newly emerged male and female mosquitoes were placed in separate screened cages to prevent mating taking place. A 10% sucrose solution was available for adults to feed on and no blood meal was offered to the females at this stage. Three day old male and female mosquitoes were exposed separately to 0.75% permethrin for 1 h. Knockdown was recorded after 1 h exposure and final mortality was recorded 24 h post exposure (WHO, 1998). Each individual was scored as either alive or dead and provided with a 10% sucrose solution. The insecticide exposure procedure is described in Chapter 3. The control consisted of mosquitoes from the susceptible strain (FANG) exposed to untreated

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papers. The FANG strain was used as control because it is fully susceptible to pyrethroid insecticides. Twenty-five *An. funestus* mosquitoes per tube were used for each replicate. Four replicates per sex were exposed.

Male and female permethrin survivors were placed in the same cage and left to mate. At age 10 days, the female mosquitoes were blood-fed. All the females that were alive were isolated individually in glass tubes lined with moist filter paper for egg laying. Females were blood-fed three times a week. The number of females that laid eggs and number of eggs laid by each female as well as the number of eggs that hatched was recorded daily. Each parameter tested was compared against the values obtained for the unexposed FUMOZ-R colony.

2.3 DATA ANALYSIS

Statistical software used in the analysis of data was Stata 8® (Statacorp, College Station, Texas) and Statistix 7®. The results were interpreted at 95% confidence (two-sided).

Tukey's students t test (P=0.05) was used to determine whether the mean number of eggs, larvae, pupae and adults per female differed significantly between the resistant and susceptible strains. Tukey's t test was also used to determine whether the sex ratios (percentage of females produced), mean developmental times by stage and

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survival through all life stages varied significantly between the resistant and susceptible strains.

Pearson's chi-square was performed in order to compare differences in the mating success, fertility rate and sterility rate between the resistant and the susceptible strains.

A log rank test was employed in order to assess a comparison of survivorship between the two strains.

2.4 RESULTS

The results of this chapter have been accepted for publication in *Bulletin of Entomological Research*. A copy of the galley proof is attached in Appendix B. A detailed description of the results obtained and discussion is given below.

2.4.1 Reproductive characteristics

2.4.1.1 Egg, larvae, pupae and adult production

The overall and mean number of eggs, larvae, pupae and consequently adults produced was greater in the resistant strain (FUMOZ-R) than in the susceptible strain (FANG) (Table 2.1 and 2.2). The mean no. of eggs/female laid by FUMOZ-R was

80.0 with a clutch size ranging between 20 and 107 while FANG laid a mean of 73.0 eggs/female with a clutch size ranging between 28 and 111.

The FUMOZ-R strain produced a significantly higher mean number of larvae (P=0.0258) and adults (P=0.0374) than the FANG strain (Table 2.1). The number of pupae produced did not differ significantly between the strains (P=0.1). The absolute numbers of each strain entering or dying at each stage are given in Table 2.2. The sex ratio (percentage of females) of adult progeny was 53% (750/1405) for FUMOZ-R and 48% for FANG (519/1072) (P=0.04) (Table 2.2). Although the FUMOZ-R sample produced more females than males, there was no statistically significant difference between the two (P=0.34). Conversely, the sample of FANG produced more males than females although the difference was not statistically significant (P=0.77).

Table 2.1: Reproductive characteristics for the *An. funestus* pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains. P values were determined by t tests using the mean and standard error (s.e.).

	FUMOZ-R	**FANG**	P
Mean no. of eggs/female	80.0 ± 6.5	73.0 ± 8.6	> 0.05
Mean no. of larvae/female	63.2 ± 4.7	46.5 ± 5.5	0.0258*
Mean no. of pupae/female	49.7 ± 4.6	37.6 ± 5.6	> 0.05
Mean no. of adults (female)	25 ± 2.3	17.3 ± 2.8	0.0374*
Mean no. of adults (male)	21.8 ± 2.4	18.5 ± 2.9	> 0.05

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* = FUMOZ-R and FANG means significantly different at 95% confidence using 2-sample t tests.

Table 2.2: Stage specific survivorship of pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains of *An. funestus*.

Stage	Number entering stage		Number dying in stage		Proportion dying in stage	
	FUMOZ-R	**FANG**	**FUMOZ-R**	**FANG**	**FUMOZ-R**	**FANG**
Eggs	2401	2190	506	794	0.21	0.36
Larvae	1895	1396	403	268	0.21	0.19
Pupae	1492	1128	87	56	0.06	0.05
Female adults	750	519	-	-	-	-
Male adults	655	554	-	-	-	-
Total adults	1405	1072	-	-	-	-

2.4.1.2 Pre-adult survivorship

A greater percentage of the eggs of the FANG strain did not hatch compared to that of FUMOZ-R (P=0.0191). However, no significant difference was recorded between the proportions that died during the larval (P=0.8084) and pupal stages (P=0.3946) in the two strains (Table 2.2).

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Table 2.3 shows the mean percentage survival survival through each life stage of pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains of *An. funestus*. The proportion of eggs that hatched was significantly higher in FUMOZ-R (81.5%) than in FANG (66.9%) (P=0.02) (Table 2.3). No significant difference was recorded between the proportions of larvae pupating and pupae emerging as adults between the two strains (Table 2.3). However, the percentage of egg-to-adult survivorship was greater in FUMOZ-R than in FANG (P=0.0489). The percentage of adults produced based on number of larvae (P=0.6659) and pupae (P=0.3948) produced was not statistically different between the two strains (Table 2.3).

Table 2.3: Mean (± s.e.) survival through each life stage of *An. funestus* pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains. P values were determined by t tests using the mean and s.e.

	FUMOZ-R	**FANG**	P
% Egg hatching	81.5 ± 3.5	66.9 ± 4.9	0.0191*
% Larvae pupating	78.5 ± 3.4	79.8 ± 4.0	> 0.05
% Pupae emerging	93.5 ± 1.6	95.2 ± 1.3	> 0.05
% Survivorship based on the no. of eggs produced	61.6 ± 4.6	49 ± 4.3	0.0489*
% Survivorship based on the no. of larvae produced	73.7 ± 3.5	76 ± 4.0	> 0.05
% Survivorship based on the no. of pupae produced	93.5 ± 1.6	95.2 ± 1.3	> 0.05

(*) indicates FUMOZ-R and FANG means significantly different at 95% confidence using 2-sample t tests.

2.4.1.3 Adult survivorship

There was no statistical difference in adult survivorship between the males and females within each strain. Females of the FANG strain showed a higher rate of survival than the corresponding male cohort during the first 31 days (Figure 2.1). Males of the FUMOZ-R strain survived slightly longer than the corresponding female cohort during the period day 3 to day 47. However, a few females survived to day 64 while all the males had died by day 55 (Figure 2.2). There was no statistical

difference in length of adult survivorship between females from the two strains (P=0.2487) (Figure 2.3). However, during the period between day 7 and day 37 females of the FANG strain showed a significantly higher rate of survival than those of the FUMOZ-R strain (P=0.001). A comparison between the two male cohorts (Figure 2.4), showed a significantly higher rate of survival in FUMOZ-R (P=0.0316). The mean longevity of the females and males of FUMOZ-R and FANG did not differ significantly between and within the two strains (Table 2.4).

Figure 2.1: Adult male and female survivorship curves for the pyrethroid susceptible strain (FANG) of *An. funestus*.

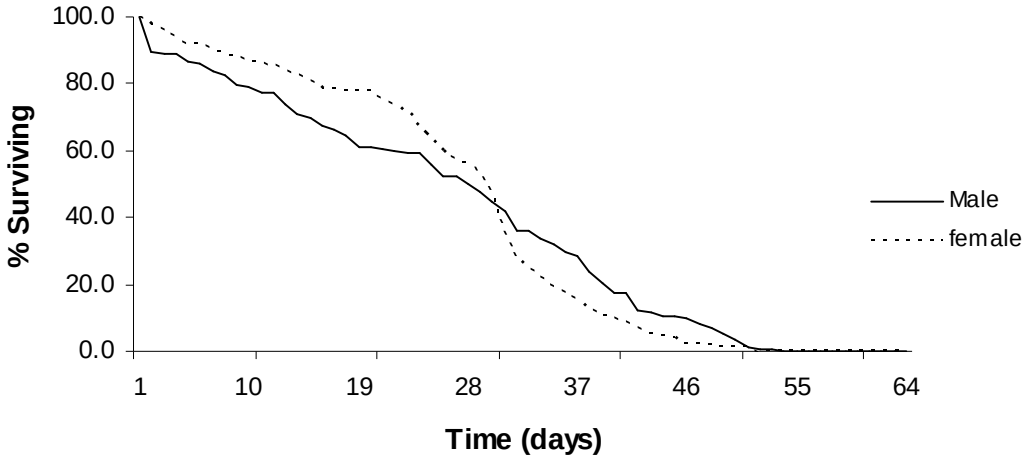

Figure 2.2: Adult male and female survivorship curves for the pyrethroid resistant strain (FUMOZ-R) of *An. funestus*.

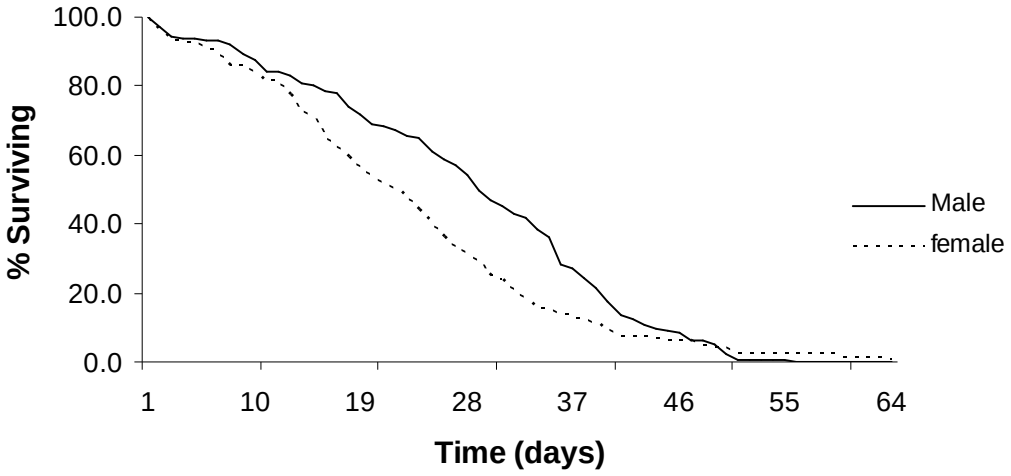

Figure 2.3: Adult female survivorship curves for the pyrethroid susceptible (FANG) and resistant (FUMOZ-R) strains of *An. funestus.*

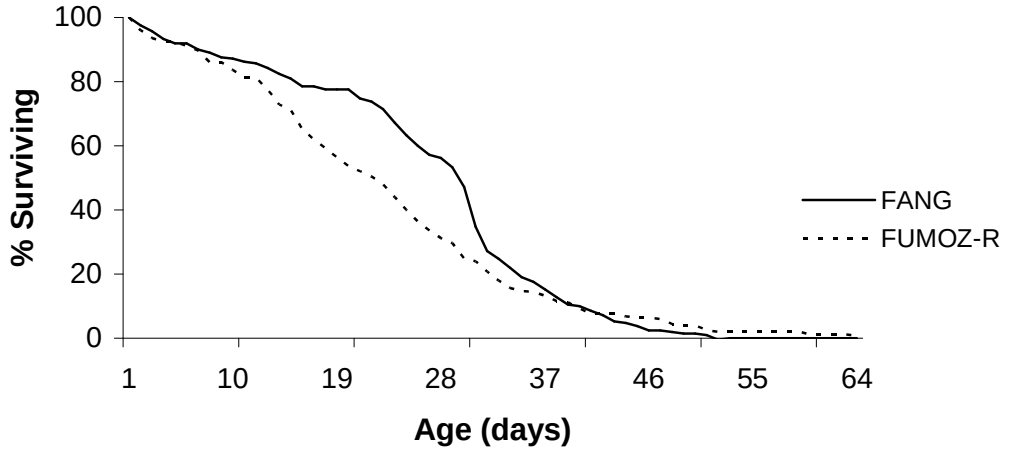

Figure 2.4: Adult male survivorship curves for the pyrethroid susceptible (FANG) and resistant (FUMOZ-R) strains of *An. funestus.*

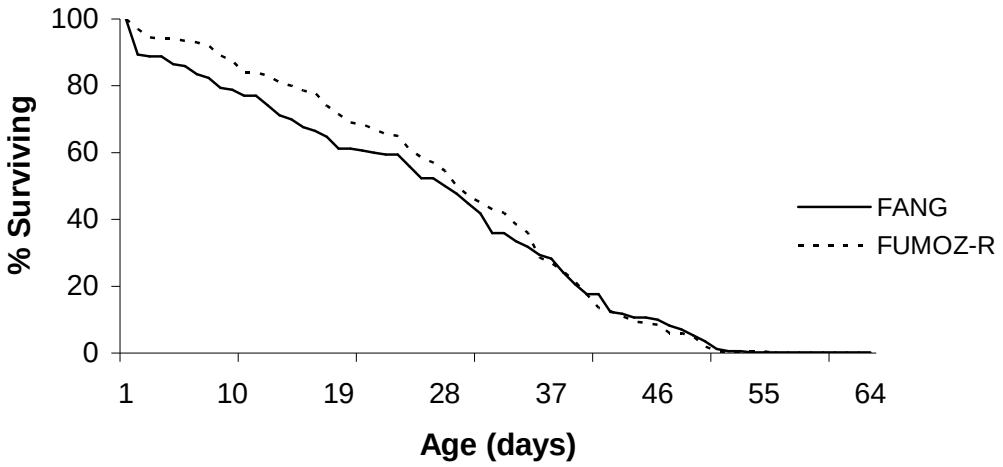

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Table 2.4: Reproductive characteristics and developmental time (in days) for the *An. funestus* pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains. Ranges (minimum–maximum) are given in parentheses. P values were determined by t tests using the mean and s.e. values.

	FUMOZ-R	**FANG**	P
Female adult longevity	48.6 ± 5.5 (39 – 64)	49.6 ± 1.9 (43 - 53)	> 0.05
Male adult longevity	48.6 ± 2.9 (40 – 57)	48.6 ± 2.2 (40 - 52)	> 0.05
Days to first egg production	15.8 (11 - 20)	15.7 (11 - 22)	> 0.05
Egg hatch to adult emergence (♀)	15.9 ± 0.3 (13 – 19)	14.9 ± 0.2 (13-17)	0.0127*
Egg hatch to adult emergence (♂)	15.8 ± 0.3 (13 – 20)	14.7 ± 0.3 (10-17)	0.0085*
Egg to adult emergence (♀)	17.8 ± 0.3 (15 – 21)	17.3 ± 0.2 (16 - 21)	> 0.05
Egg to adult emergence (♂)	17.8 ± 0.3 (15 – 22)	17.2 ± 0.2 (15 - 20)	> 0.05
F0 to F1 adult (♀)	33.6 ± 0.7 (26 – 41)	33.1 ± 0.5 (29 – 40)	> 0.05
F0 to F1 adult (♂)	33.6 ± 0.7 (26 – 42)	32.9 ± 0.4 (29 – 40)	> 0.05
No. of blood meals prior to first egg production	3 ± 0.2 (2 – 4)	3 ± 0.2 (2 – 6)	> 0.05

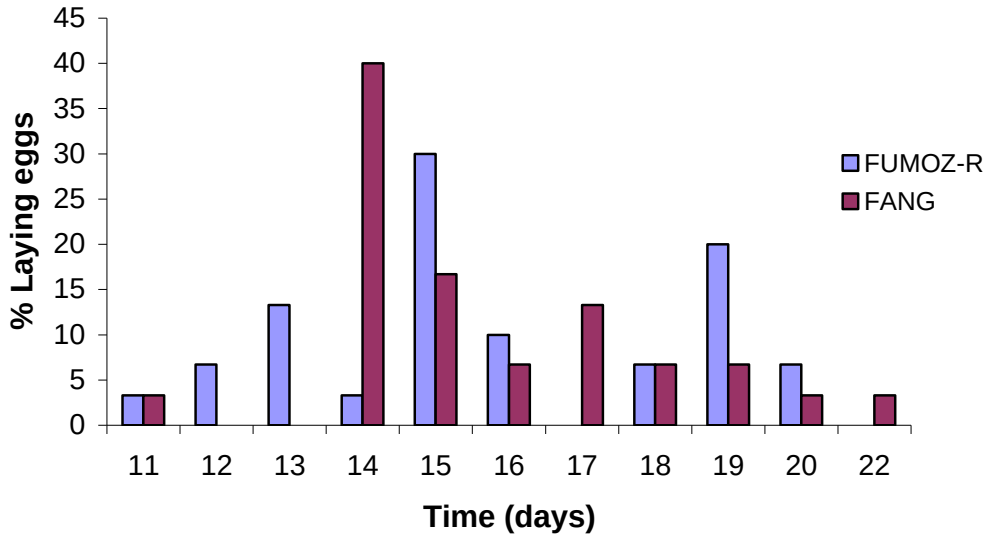
(*) indicates FUMOZ-R and FANG means significantly different at 95% confidence using 2-sample t tests.

2.4.2 Developmental time (in days)

2.4.2.1 Adult emergence to first egg production

Egg laying (following a 10 day mating period) occurred on average at 15.8 days and 15.7 days following adult emergence for the FANG and FUMOZ-R strains respectively (Table 2.4). The majority of FUMOZ-R females laid eggs on day 15 while the majority of FANG females laid eggs on day 14 (Figure 2.5).

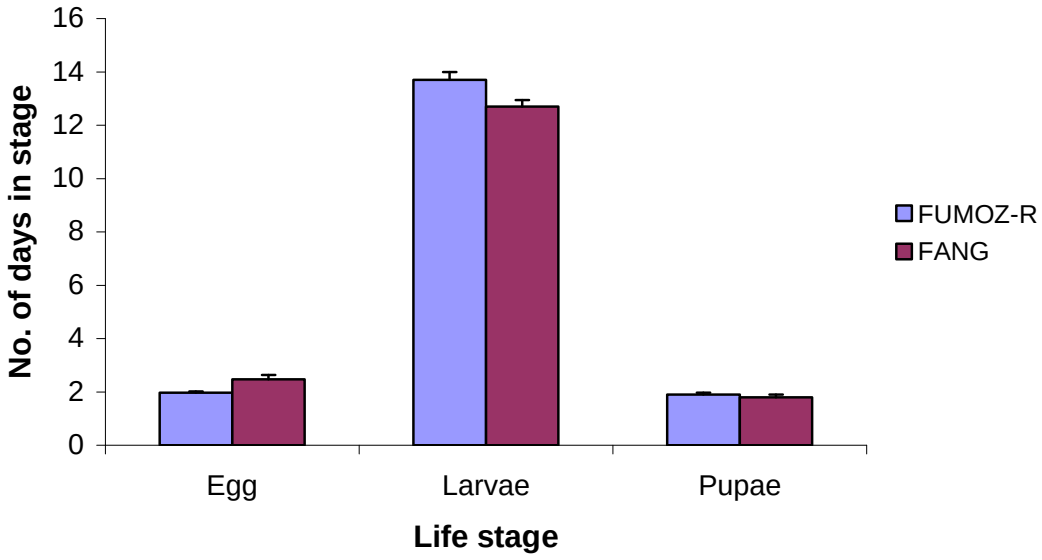
Figure 2.5: Age at oviposition of pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains of *An. funestus*.



2.4.2.2 Oviposition to egg hatching

The mean time from oviposition to egg hatching was significantly longer in the FANG strain (2.5 days) than in the FUMOZ-R strain (2 days) (P=0.0068), with a range of 1 - 3 days for FUMOZ-R and 1 - 6 days for FANG (Figure 2.6). Eggs from 27 families (90%) hatched 2 days following oviposition while 2 families (6.7%) hatched after 1 day and 1 family (3.3%) hatched after 3 days. Results for the FANG strain showed that egg batches from 18 families (60%) and 9 families (30%) hatched after 2 days and 3 days respectively while 1 egg batch hatched after 1 day, 1 after 4 days and 1 after 6 days.

Figure 2.6: Mean (±s.e.) developmental time (in days) spent at pre-adult stages of pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains of *An. funestus*.



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2.4.2.3 First instar larva to pupa

The mean developmental time from first instar larva to pupa was significantly longer in FUMOZ-R (13.7 days) than in FANG (12.7 days) (P=0.011), with a range of 11 - 17 days for FUMOZ-R and 8 - 15 days for FANG (Figure 2.6). The majority (80%) of larvae from FUMOZ-R families pupated between day 12 and 15 following oviposition while the majority of larvae from FANG families (93.4%) pupated between day 11 and 14 following oviposition.

2.4.2.4 Pupa to adult

The mean time from pupa to adult stage (30 families per strain) was 1.9 days for FUMOZ-R and 1.8 days for FANG (Figure 2.6). No significant difference was recorded in developmental time from pupa to adult between strains and ranged from 1 - 3 days in both strains.

2.4.2.5 Egg hatch to first adult emergence

The mean time taken from egg hatch to first adult emergence was not statistically different between females (P=0.9379) and males (P=0.7023) of both FUMOZ-R and FANG (Table 2.4). Table 2.5 details the mean developmental time in days from egg hatch to first adult emergence across all families of FUMOZ-R and FANG strains.

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Table 2.5: Developmental time (in days) from egg hatch to first adult emergence for families of pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains of *An. funestus*. n = number of families and percentages are given in parentheses.

FUMOZ-R			FANG		
	Females	**Males**		**Females**	**Males**
Day	**n (%)**	**n (%)**	**Days**	**n (%)**	**n (%)**
13	2 (6.7)	2 (6.7)	10	-	1 (3.3)
14	6 (20.0)	6 (20)	13	3 (10.3)	3 (10)
15	4 (13.3)	3 (10)	14	9 (31)	7 (23.3)
16	7 (23.3)	8 (26.7)	15	8 (27.6)	10 (33.3)
17	6 (20)	8 (26.7)	16	6 (20.7)	7 (23.3)
18	3 (10)	2 (6.7)	17	3 (10.3)	2 (6.7)
19	2 (6.7)	-	-	-	-
20	-	1 (3.3)	-	-	-
Total	30	30	Total	29	30

2.4.2.6 Egg to first adult emergence

The mean time from egg to first adult emergence did not differ significantly between females (P=0.24) and males (P=0.15) of both strains (Table 2.4). Nor did the time from egg to first adult emergence differ between the males and females within FUMOZ-R (P=0.94) and FANG (P=0.73). Table 2.6 details the mean developmental time in days from egg to adult emergence across all families of FUMOZ-R and FANG. The majority of FUMOZ-R females (23.3%) and males (26.7%) required 18 days from egg to first adult emergence while the majority of FANG females (34.5%) and males (30%) required 16 days.

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Table 2.6: Developmental time (in days) from egg to first adult emergence for families of pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains of *An. funestus*. n = number of families and percentages are given in parentheses.

FUMOZ-R			FANG		
	Females	**Males**		**Females**	**Males**
Days	**n (%)**	**n (%)**	**Days**	**n (%)**	**n (%)**
15	4 (13.3)	4 (13.3)	15	-	1 (3.3)
16	4 (13.3)	4 (13.3)	16	10 (34.5)	9 (30)
17	4 (13.3)	3 (30)	17	7 (24.1)	7 (23.3)
18	7 (23.3)	8 (26.7)	18	6 (20.7)	9 (30)
19	6 (20)	7 (23.3)	19	5 (17.2)	3 (10)
20	2 (6.7)	3 (10)	20	-	1 (3.3)
21	3 (10)	-	21	1 (3.4)	-
22	-	1 (3.3)	-	-	-
Total	30	30	Total	29	30

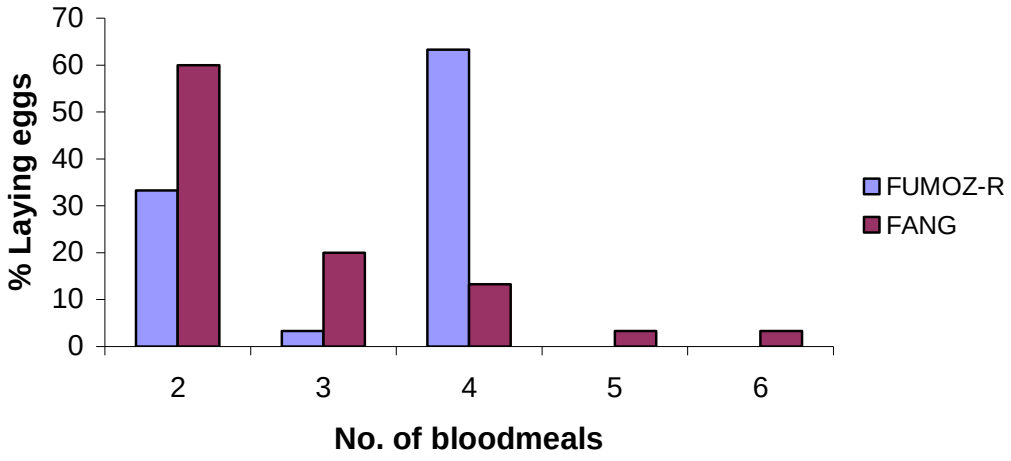
2.4.2.7 Generation time

Generation times (adult to adult) did not differ significantly between the FUMOZ-R and FANG strains (Table 2.4). Generation time did not differ significantly between females and males of FANG (P=0.8046). Similarly, there was no difference between the females and males of FUMOZ-R (P=0.9740).

2.4.3 Blood meals required for egg production

The number of blood meals required by females of each strain in order to lay eggs was not significantly different (Table 2.4). Figure 2.7 details the percentage of females of FANG and FUMOZ-R strains that laid eggs after taking successive blood meals. The majority of FANG females (60%) and FUMOZ-R females (63.3%) laid eggs after 2 and 4 successive blood meals respectively.

Figure 2.7: Frequency distribution of the percentage of females of pyrethroid susceptible (FANG) and resistant (FUMOZ-R) strains of *An. funestus* that laid eggs after taking successive blood meals.



2.4.4 Egg fertilization and viability

The dissection of spermathaecae revealed that 52.6% (n=156) of FANG females were fertilized compared to 56.8% (n=162) of FUMOZ-R females. There was no statistical difference between them (χ^2=0.57, P=0.32). The proportion of females that successfully produced eggs was significantly higher in the FUMOZ-R strain (χ^2=9.05, P=0.003). Forty-three percent (n=30) and 23.3% (n=30) of females from FUMOZ-R and FANG produced eggs respectively. Complete failure to hatch was recorded in 28.6% (n=7) of FANG families and 7.7% (n=13) of FUMOZ-R families, showing a significant difference (χ^2=14.62, P<0.001).

2.4.5 Effect of insecticide exposure on fecundity and fertility of pyrethroid resistant *An. funestus*

Of the 100 females and 100 males exposed to permethrin for 1 h as described in the methodology, 98 females and 87 males survived. They were allowed to mate and, on day 10, the surviving females (n=64) were placed individually in glass vials lined with moist filter paper in order to lay eggs.

Of the 64 exposed females isolated, 28 (43.8%) laid eggs and 36 (56.2%) died without laying eggs. Of the 28 females that laid eggs, 18 (64.3%) families hatched and 10 (35.7%) families did not hatch. The fecundity rate (proportion of females that successfully produced eggs) did not differ significantly between the exposed (43.8%) and the unexposed FUMOZ-R samples (43.3%) (χ^2=0.02, P=0.8866). The sterility

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rate (complete failure to hatch) for the exposed females (35.7%) was significantly higher than the 7.7% for the unexposed FUMOZ-R females (χ^2=68.06, P<0.001).

The mean number of eggs produced per exposed female was significantly lower than that of the unexposed FUMOZ-R females (54 versus 80) (P=0.0017). Further, the mean number of larvae produced per exposed family was significantly lower than that of the unexposed FUMOZ-R families (28.4 versus 63) (P<0.001).

2.5 DISCUSSION

This is the first study presenting life-table data for *Anopheles funestus*. In addition, it presents the first report on the possible fitness cost and advantage associated with pyrethroid resistance in this species.

2.5.1 Fecundity and fertility

The pyrethroid resistant strain of *An. funestus* showed a higher fertility than the susceptible strain. On average, resistant females were 1.1 times more fecund than susceptible females. The proportion of females that laid eggs and the proportion of viable eggs produced were higher in the resistant strain than in the susceptible strain. In this study the average number of eggs recorded per female was 80 for the resistant and 73 for the susceptible strain. The mean clutch size varied between 20 - 107 for the resistant strain and 28 - 111 for the susceptible strain. Hocking & MacInnes

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(1948) reported that the average number of eggs laid in each batch of *An*. *funestus* was 123. Detinova and Gillies (1964) found mean clutch size to vary between 83 and 139. It is also worth noting that during the egg production experiment to measure the fecundity and fertility rate, the mortality in the susceptible strain was high and experiments had to be repeated three times in order to confirm the results obtained (which remained consistent in each case). This might be an indication of increased relative fitness of the resistant strain. These data indicate that the resistant strain of *An. funestus* had an advantage in terms of higher fertility, proportion of females laying eggs and lower sterility rate compared with susceptible females, and showed no fecundity deficiency associated with its resistance to pyrethroids.

The females used to study fecundity and fertility had not been exposed to pyrethroids prior to the experiment in order to eliminate the possible effects of sublethal doses on adult reproduction. In this study, resistant females exposed to insecticide prior to egg laying produced fewer eggs and had a higher sterility rate than the unexposed resistant females. This indicates that sublethal dose of insecticide affected the fecundity and fertility of the females.

2.5.2 Developmental time

Time from oviposition to egg hatching was longer for the susceptible strain than the resistant strain. However, the developmental time from larval to pupal stage was

longer for the resistant strain relative to the susceptible strain. There was no difference between the developmental time from pupa to adult and in the generation time (F0 adult to F1 adult) for both strains. el-Khatib & Georghiou (1985) demonstrated that organophosphate resistant *C. quinquefasciatus* mosquitoes required a longer pupation period and had a delayed female emergence. Differences in development time between insecticide resistant and susceptible populations are generally considered to have more effect on the reproductive potential of individuals than differences in fecundity (Roush & Croft, 1986). This is because it has been proposed that longer larval development time may be expressed as a fitness cost (Lenormand *et al*., 1998)

The developmental time recorded for both resistant and susceptible strains of *An. funestus* agreed with previous reports (see Gillies & De Meillon, 1968). The life cycle from egg to adult was longer in the resistant strain than in their susceptible counterparts. The data presented here showed that the life cycle was 17.8 days for both resistant females and males (egg stage 1 - 3 days, larval stage 11 - 17 days and pupal stage 1 - 3 days) and 17.3 and 17.2 days for the susceptible females and males respectively (egg stage 1 - 6 days, larval stage 8 - 15 days and pupal stage 1 - 3 days). The generation time (adult to adult) was 34 and 33 days for the resistant and susceptible strains respectively. Development time of 16 days from egg to adult at 27°C has been recorded in the laboratory for *An. funestus* (see Gillies & De Meillon, 1968). According to Hocking & MacInnes (1948), the development of *An. funestus* eggs lasts 2 - 3 days. The development of larvae under field conditions has been

found to last 15 days at 23.4°C and in the laboratory, 15 days at 26.9°C, and the pupal stage lasting 2 - 3 days. This gives a total development time from egg to adult of 21 days (see Gillies & De Meillon, 1968). In Mauritius, Jepson *et al.* (1947) concluded that under natural conditions full development was rarely completed in less than 21 days and that in the laboratory at 24°C the total life-cycle varied from 21 to 30 days. Service & Oguamah (1958) reported that the duration from eggs to pupae was 21 days at 50 - 85°F. Hunt *et al.* (2005) reported a life cycle from egg to adult of 36 days and adult survivorship of approximately 6 weeks for *An. funestus*.

2.5.3 Survivorship

The FUMOZ-R resistant strain showed a significantly higher survivorship in the egg stage (which could be due to higher egg viability) but had a slightly higher (but not significant) mortality in the larval and pupal stage than the FANG susceptible strain. Further, the resistant strain showed a greater percentage of adult emergence based on the initial number of eggs. The higher overall yield of adults by the resistant strain in *An. funestus* may have been favoured by the high egg production of resistant females.

The sex ratio (expressed as percentage of females) of the resistant strain was higher than that of the susceptible strain. The resistant strain produced more females than males (750 versus 655) while the reverse was the case with the susceptible strain (519 females versus 554 males). However, following the assumptions cited in Agnew *et al.* (2000) there could be two additional explanations for this: firstly, the mortality of

male and female pre-adult stages was the same, but the original sex ratio was biased in favour of either females (for the resistant strain) or males (for the susceptible strain); secondly, the original sex ratio of males and females was equal, but pre-adult stage mortality was biased toward either males (for the resistant strain) or females (for the susceptible strain).

Longevity is one of the most important factors contributing to the ability of malaria vectors to transmit malaria parasites (Gillies & De Meillon, 1968). The longevity of some females of the resistant strain was longer than that of the susceptible strain. Longevity recorded in the laboratory would almost certainly be higher than under natural conditions because access to a blood meal source and to additional food (10% sucrose solution) could be limited in nature. Another factor could be the presence of predators in nature. The mosquitoes used in this study were provided with sucrose daily and the females were blood-fed three times a week.

Environmental factors such as temperature, nutrition, and larval density affect developmental rates and survivorship of different mosquito life stages (Reisen *et al*. 1984; Lyimo *et al.*, 1992; Agnew *et al.* 2000). Temperature is important in behaviours such as host seeking as well as in blood feeding and development (Kirby & Lindsay, 2004). In this study, temperature was controlled (25 ± 3^{o}C), but nutrition (amount of food per larva) and larval density were not. Larvae from each family were kept in approximately the same volume of water with constant surface area large enough to avoid intense competition for food. The amount of food supplied per larval

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bowl depended on the larval density and larvae were checked constantly and re-fed once the food on the water surface was reduced. Thus, food provision was ample and unlikely to have been a limiting factor for larval survival and growth.

The loss of fitness that often accompanies an increase in homozygosity of recessive semi-lethal genes due to inbreeding is generally referred to as inbreeding depression and can reduce mosquito fitness during laboratory colonization (Munstermann, 1994; Bijlsma *et al*., 1999; Armbruster *et al*., 2000). It can reduce fitness by causing decreased fertility, lower fecundity, increased sterility, decreased mating success and slower development (Bijlsma *et al*., 2000).

Few differences in biological fitness were recorded between the pyrethroid resistant and susceptible strains of *An*. *funestus*. There was no uniform negative effect across all fitness parameters between strains. Negative performance in one parameter can conceivably be balanced by positive performance in another. Further, certain parameters can have knock-on effects. For example, the susceptible strain exhibited reduced fecundity that was matched with a decrease in adult production relative to the resistant strain. Thus, results obtained suggest that pyrethroid resistance in southern African *An. funestus* does not incur any loss of fitness. Reduced fitness is not always linked to resistance and there are several cases where differences in fitness between resistant and susceptible strains are found to be small and some cases where the resistance strain seems to have an advantage (Varzandeh *et al.*, 1954; Roush & Hoy, 1981; Heather, 1982; Haubruge & Arnaud, 2001).

Absence of a fitness cost associated with insecticide resistance in some insecticide resistant strains have been associated with the pleiotropic effect of (a) selection of modifier alleles at other genes that minimise the adverse effects of resistance alleles in the absence of insecticides (as has been reported for the blowfly *Lucilia cuprina* for the costly diazinon resistant allele at the *Rop-1* locus (Clarke & McKenzie, 1987; Arnaud *et al*., 2002)); or (b) due to the replacement of a costly resistance allele by a less costly one at the same locus (as suggested for the replacement of *Ester*^{1} by *Ester*^{4} at the *Ester* locus of *C. pipiens* (Bourguet *et al*., 2004)) giving the resistant strain a reproductive advantage compared to the susceptible strain. However, the presence of a modifier gene or replacement allele is not known in *An. funestus.* There is also a relationship between the mechanism of resistance and fitness in the absence of insecticide selection pressure (Roush & McKenzie, 1987). Enhanced quantities of esterases may be associated with a reproductive disadvantage. Conversely, presence of altered acetylcholinesterase, increased monooxygenase detoxification, malathion-specific carboxylesterases and *kdr* seem to be associated with little or no fitness disadvantage (Roush & McKenzie, 1987). Pyrethroid resistance in the *An. funestus* strain used here has been shown to be metabolic, essentially based on the overproduction of monooxygenases (Brooke *et al.*, 2001).

The fact that the pyrethroid resistant strain (FUMOZ-R originating from southern Mozambique) and pyrethroid susceptible strain (FANG originating from southern Angola) do not share the same genetic background requires careful consideration. A

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susceptible strain could not be selected from the baseline colony from which the resistant strain was derived because of the high level of resistance present in it. However, various studies have shown that the two strains are genetically similar. Analyses of *Msp I* (HPa II) digests of a 400 bp fragment of domain 3 (D3) of the 28S gene in the rDNA of *An. funestus* from samples collected in west, central, east and southern Africa show clear evidence of population structuring (Garros *et al*., 2004). However, samples from southern Angola, the FUMOZ laboratory colony, southern Mozambique and South Africa clustered together as the MW-Type, suggesting that they are genetically similar (Koekemoer *et al.*, 2006). These results are re-inforced by cross-mating experiments between the FUMOZ and FANG strains demonstrated in Chapter 3 of this study and also by Ntomwa (2004) who showed that hybrid F1 progeny of both sexes are fertile and viable. However, laboratory crosses between the different types (M/W/MW-types) have never been recorded and it is unclear what the importance of population structuring is in *An. funestus*.

Future studies can be carried out to consolidate these findings. Firstly, the resistance allele(s) (FUMOZ-R) can be backcrossed to the susceptible allele(s) (FANG) and the fitness comparison with FANG repeated so that only the effects of the resistance/susceptible alleles would be operating. Secondly, caged populations with an initial 1:1 ratio of the two strains could be set up and bred for a few generations with observation of any rise or fall in the frequency of resistance without insecticide treatment.

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CHAPTER 3

INHERITANCE OF PYRETHROID RESISTANCE IN *AN. FUNESTUS*

3.1 INTRODUCTION

The mode of inheritance of resistance to various insecticides has been studied in various mosquito species as detailed in Chapter 1. The aim of this chapter was to determine the mode of inheritance of resistance genes in an *Anopheles funestus* pyrethroid resistant strain and to monitor the stability of resistance expression in the resistant strain in the absence of insecticide selection pressure through several generations.

3.2 MATERIALS AND METHODS

3.2.1 Laboratory strains of *An. funestus*

Three laboratory colonies of *An. funestus* were used for this study (FUMOZ, FUMOZ-R and FANG). FUMOZ is the baseline colony from which FUMOZ-R was selected. The FUMOZ colony has not been selected for resistance to any insecticide since it was established in 2001. Details of the FUMOZ-R and FANG strains as well as mosquito rearing conditions are given in Chapter 2.

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3.2.2 Insecticides and test kits

The WHO test kits and insecticide treated papers used in this study were obtained from University Sains Malaysia, Penang (WHO, 2001b). All batches of insecticide treated papers used were tested on a laboratory colony of *An. funestus* (FANG) because it is known to be fully susceptible to all insecticides.

3.2.3 Determination of inheritance of insecticide resistance genes

Cross mating experiments were undertaken between the pyrethroid resistant (FUMOZ-R) and susceptible (FANG) colonies. These colonies are maintained in separate rooms to prevent cross contamination. Adult male and female mosquitoes were separated at emergence to prevent mating prior to crossing.

Each cross was left in a cage to mate for 10 days during which time females were offered blood meals three times a week. Egg plates were placed in each cage two times a week. After egg laying, eggs were placed in larval rearing bowls containing approximately 200 ml of distilled water. The water level was kept constant by adding water when necessary. Larvae were reared through to adults in the same insectary (as described in Chapter 2) under the same conditions in order to minimise environmental variation.

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Reciprocal crosses between the parental strains (B1 and B2) as well as several backcrosses to the parental strains (H1; H2; H3 and H4) and intercrosses of the progeny of the reciprocal crosses (B1F2 and B2F2) were carried out as shown in Table 3.1. These were carried out in order to determine the following: (a) the number of genes involved in resistance; and (b) the resistance inheritance mechanism (sex-linked, dominant, co-dominant or recessive). In all experiments, mass matings were carried out to maximise progeny numbers.

To evaluate sex linkage, reciprocal cohorts of F1 hybrid progeny were isolated for exposure to 0.75% permethrin for 1 h (WHO, 1998) and the results compared. The remaining F1 hybrids were backcrossed to either the resistant or the susceptible parental strains in order to determine the phenotypic expression of resistance (Table 3.1). Owing to the unavailability of suitable numbers of F1 progeny as well as the absence of a statistically significant difference between reciprocal crosses, only B1 was used for the backcrosses. A cohort of each of the F1 hybrid progeny (B1 and B2) was inbred in order to obtain an F2 generation for each (B1F2 and B2F2 respectively). These F2 progeny were exposed to 0.75% permethrin for 1 h in order to test assumptions concerning the number of loci influencing resistance. The susceptibility of the progeny of each of the crosses, intercrosses and backcrosses to 0.75% permethrin was analyzed separately. Details of the insecticide susceptibility tests are given below.

Table 3.1: Scheme of crosses and backcrosses between the permethrin resistant (RR) and susceptible (SS) strains of *An. funestus*.

Type of cross	Name	Cross	Mother	Father
F1 Hybrid	B1	SS ♀ x RR ♂	SS	RR
	B2	SS ♂ x RR ♀	RR	SS
Backcross to RR	H1	B1 ♀ x RR ♂	SS ♀ x RR ♂	RR
	H3	B1 ♂ x RR ♀	RR	SS ♀ x RR ♂
Backcross to SS	H2	B1 ♀ x SS ♂	SS ♀ x RR ♂	SS
	H4	B1 ♂ x SS ♀	SS	SS ♀ x RR ♂
F2 Hybrid	B1F2	B1 ♀ x B1 ♂	SS ♀ x RR ♂	SS ♀ x RR ♂
	B2F2	B2 ♀ x B2 ♂	SS ♂ x RR ♀	SS ♂ x RR ♀

3.2.4 Insecticide susceptibility tests

Levels of permethrin resistance were determined for the parental (RR and SS), F1 (RS) progeny, backcross progeny and intercross progeny, by exposing samples of 2 – 3 day old mosquitoes to 0.75% permethrin for 1 h. Susceptibility tests were performed in the insectary according to WHO (1998) protocols.

The test kit (see Appendix A, Figure A.4) comprised of: plastic cylinders marked with a red dot for use as ‘exposure chambers’, i.e. for exposing mosquitoes to insecticide-impregnated papers; plastic cylinders marked with a green dot for use as ‘holding chambers’, for pre-test sorting and for post-exposure observation; slide-

units; 0.75% permethrin impregnated papers (for lining the exposure chambers) and sheets of clean paper (12 x 15 cm) for lining the holding chambers.

Twenty five to thirty *An. funestus* mosquitoes per tube were used for each replicate. Approximately four replicates per cross or backcross were performed depending on the number of progeny available. Controls consisted of mosquitoes from the fully susceptible strain (FANG) exposed to untreated papers.

Mosquitoes were introduced into each holding tube using an aspirator. The slide unit was opened and the mosquitoes were gently but firmly blown into the exposure chamber and the slide unit was closed. Exposure time was one hour and the exposure chambers were held in a vertical position during the entire exposure. The number of mosquitoes (knockdown (KD)) at the bottom of the tube was counted after 5, 10, 15, 20, 30, 40, 50 and 60 minutes. Following exposure all knocked down and surviving mosquitoes were transferred back to the holding tube and offered a 10% sucrose solution. Final mortality was determined 24 h post exposure by scoring each individual as either alive or dead.

3.2.5 Stability of resistance in the FUMOZ base colony

In order to assess the stability of insecticide resistance over time with no insecticide selection pressure, the FUMOZ base colony was maintained for sixteen generations without insecticide selection pressure. During this time, cohorts of two to three day

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old *An. funestus* from each generation of the unselected base colony (FUMOZ) were exposed to 0.75% permethrin for 1 h as previously described in order to determine the resistance level of each generation. Knockdown was recorded after 60 minutes exposure and final mortality was recorded 24 h post exposure. The number of female mosquitoes tested per generation ranged from 107 to 326. Controls consisted of 332 pyrethroid susceptible (FANG) females.

3.3 DATA ANALYSIS

Under the assumption of monofactorial and autosomal inheritance of resistance, data were tested using 1-sample t tests between observed mortalities and those expected based on simple Mendelian inheritance models (Wilson *et al.*, 1973). Tukey's students t test (P=0.05) was used to determine differences between the mortalities of the crosses. Statistical software used in the analysis of data was Statistix 7®.

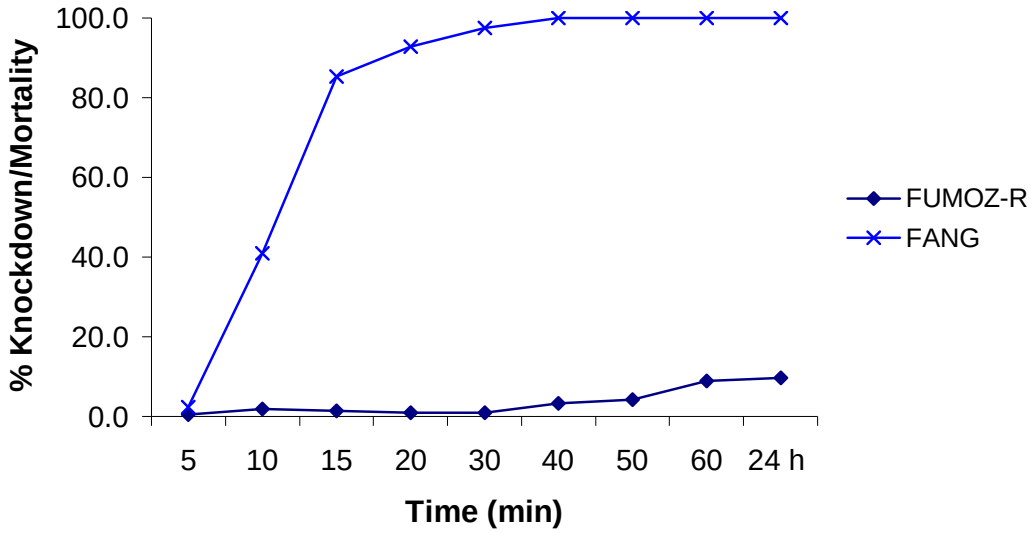
3.4 RESULTS

3.4.1 Time mortality response of parental crosses

Mean knockdown recorded for the susceptible parental strain (FANG) increased from 2.4% at 5 min to 97.5% at 30 min. Forty minutes of exposure yielded 100% knockdown (Figure 3.1). Final mortality 24 h post exposure was 100%.

Mean knockdown recorded for the resistant parental strain (FUMOZ-R) was 0.4% at 5 min and 8.8% at 60 min. Final mean mortality 24 h post exposure was 9.7% (Figure 3.1).

Figure 3.1: Percentage knockdown/mortality response for FUMOZ-R and FANG strains based on 1 h exposure to 0.75% permethrin.

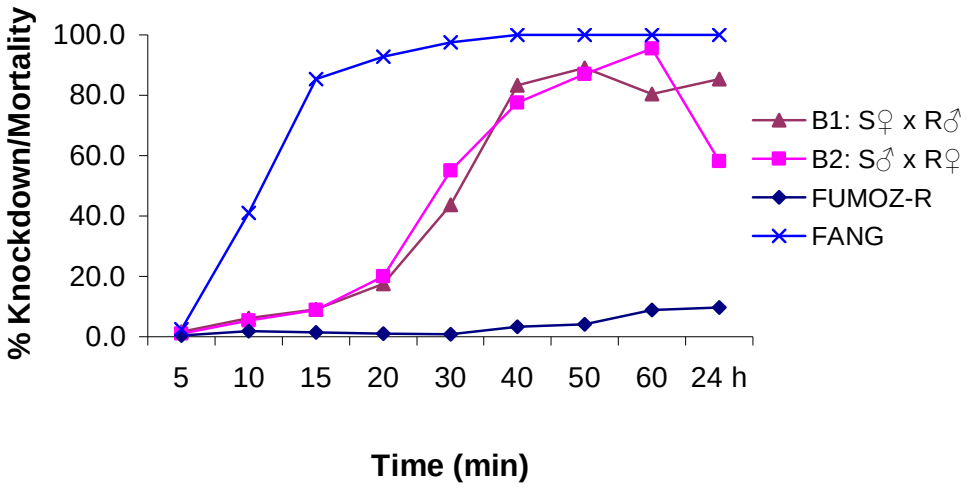


3.4.2 Time mortality response of reciprocal crosses

The mode of inheritance of permethrin resistance in *An. funestus* was determined using a single dose of permethrin (0.75% for 1 h) against F1 hybrids as well as the F2 offspring of hybrid intercrosses and backcrosses to the resistant and susceptible

parental strains. The heterozygous offspring (F1) resulting from reciprocal crosses between permethrin resistant and susceptible adults responded alike to permethrin exposure within the 60 minutes test period but showed significant differences 24 h post exposure (Figure 3.2) (2-sample t test; P=0.003). However, in both cases there were significant departures from the mortality curves illustrating the FUMOZ-R and FANG responses to permethrin exposure (1-sample t tests, $p<0.05$). The results suggest intermediate expression of the resistance phenotype in the F1 progeny and show no indication of sex linkage. The knockdown rate of both reciprocal crosses was closest to that of the resistant parental colony for the first 20 minutes but then tended towards that of the susceptible parental strain towards the end of the 1 h exposure. Knockdown after 1 h peaked at 80.5% and 95.4% for B1 and B2 respectively. However, at 24 h post exposure the mean mortality level was 85.3% for B1 and 58.1% for B2 (Figure 3.2).

Figure 3.2: Percentage knockdown/mortality response for reciprocal crosses B1 = SS ♀ x RR ♂ and B2 = SS♂ x RR♀ based on 1 h exposure to 0.75% permethrin.

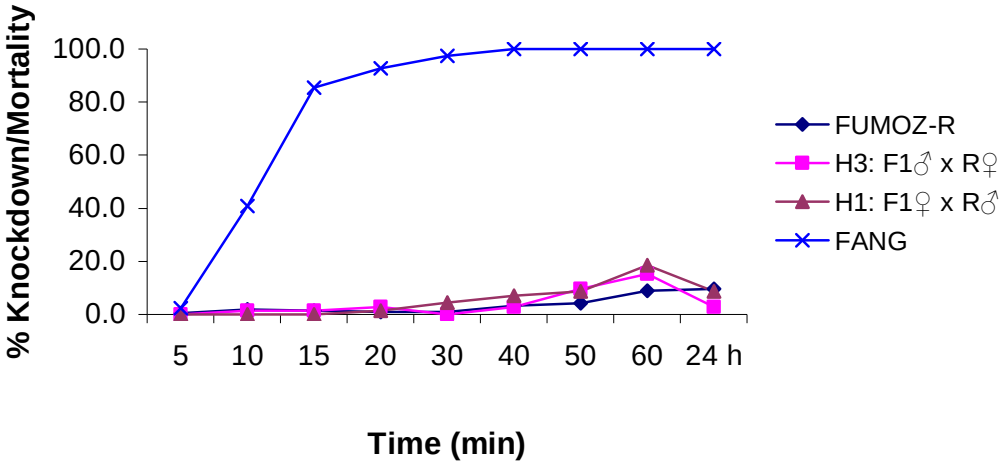


3.4.3 Time mortality response of backcrosses to the resistant parental strain

The results of backcrosses were compared against hypothetical scenarios assuming resistance to be controlled by a single genetic factor based on the principles of simple Mendelian inheritance (Wilson *et al*., 1973). Theoretically, under the assumption that resistance is monogenic and autosomal, the F2 progeny obtained by backcrossing F1 hybrid progeny (RS) to the resistant parental strain (RR) would produce genotype proportions of 50% RR and 50% RS (Tabashnik, 1991) which, assuming dominance of resistance, would yield approximately 9.7% mortality (based on the final mortality observed in the resistant parental strain) (Table 3.2). Alternatively, a recessive resistance factor would show approximately 50% mortality. At 24 h post exposure,

the results for the two backcrosses (H1 and H3) to the resistant strain were similar (P = 0.42) (Figure 3.3). The mean mortality obtained for H1 was 8.7% (1-sample t test, dominance: P=0.85, recessiveness P=0.002) and for H3 was 2.8% (1-sample t test, dominance = 0.13, recessiveness P=0.002) both of which were not significantly different from the dominant model but significantly different from the recessive model (Table 3.2).

Figure 3.3: Percentage knockdown/mortality response response for F1 progeny backcrossed to the resistant parental strain (FUMOZ-R) based on 1 h exposure to 0.75% permethrin.

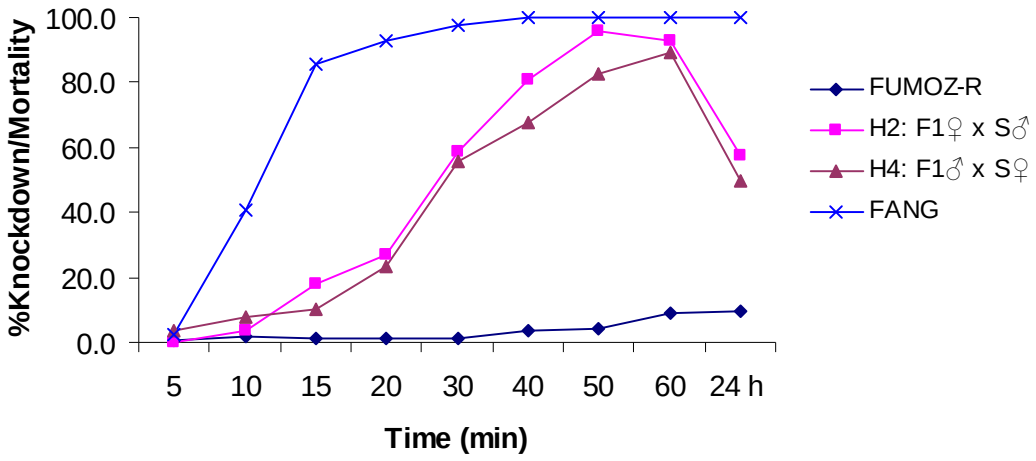


3.4.4 Time mortality response of backcrosses to the susceptible parental strain

Under similar assumptions of monogenic and autosomal control of resistance, F2 progeny obtained by backcrossing F1 hybrid progeny (RS) to the susceptible parental

strain (SS) would have produced genotype proportions of 50% SS and 50% RS (Wilson *et al.*, 1973). This scenario would have yielded approximately 50% mortality under the assumption of complete dominance or 100% mortality under the assumption of a recessive resistance factor (Table 3.2). The results for the two backcrosses (H2 and H4) to the susceptible strain were similar and showed 57.5% (H2) and 49.8% (H4) mortality (P=0.57) at 24 h post exposure indicating a tendency toward phenotypic dominance of the resistance factor (Figure 3.4). The final mean mortalities of 57.5% (H2) (1-sample t test, dominance: P=0.34 and recessiveness P=0.008) and 49.8% (H4) (1-sample t test, dominance: P=0.98 and recessiveness P=0.0003) were not significantly different from the dominant model but significantly different from the recessive model (Table 3.2).

Figure 3.4: Percentage knockdown/mortality response for F1 progeny backcrossed to the susceptible parental strain (FANG) based on 1 h exposure to 0.75% permethrin.



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3.4.5 Time mortality response of F2 (F1 intercross)

The time knockdown/mortality response obtained for B1F2 (F2 progeny of the hybrid F1 (B1) intercross) gave an exposure mean knockdown of 0% at 5 min, and 65% at 60 min. The final mortality at 24 h post exposure was 56.3% (Figure 3.5). The time knockdown/mortality response obtained for the B2F2 (F2 progeny of the hybrid F1 (B2) intercross) gave an exposure mean knockdown of 2.6% at 5 min, and 88.9% at 60 min. The final mortality at 24 h post exposure was 56.3% (Figure 3.5). The final mortalities of B1F2 and B2F2 were not significantly different from each other (P=0.4759). The assumptions of a single, dominant controlling factor would have yielded approximately 25% mortality whilst a single recessive factor would have yielded approximately 75% mortality (Davidson, 1958). The final mortality of B1F2 (56.3%) (1-sample t test, dominance: P=0.1112; recessiveness: P=0.2719) was not significantly different from either model (Table 3.2). However, the final mortality recorded for B2F2 (69.4%) (1-sample t test, dominance: P=0.0098; recessiveness: P=0.6325) was significantly different from the dominant model but not significantly different from the recessive model suggesting a tendency toward a recessive phenotypic expression (Table 3.2).

Figure 3.5: Percentage knockdown/mortality response for F2 progeny based on 1 h exposure to 0.75%

permethrin.

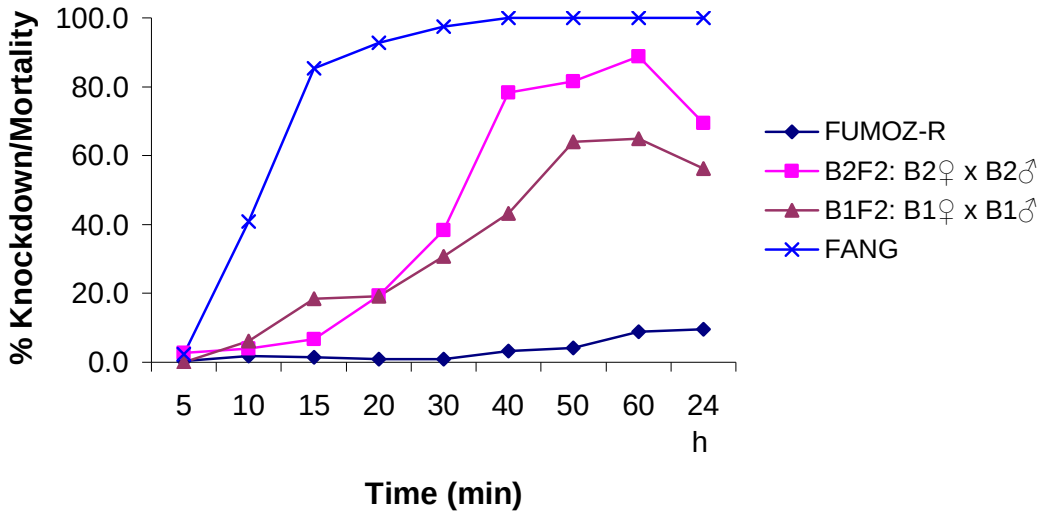

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Table 3.2: Comparison of mean (± s.e.) observed and expected mortality of progeny resulting from crosses, intercrosses and backcrosses to the resistant (FUMOZ-R) and susceptible (FANG) parental strains. Results are expressed 24 h post exposure following exposure to 0.75% permethrin for 1 h.

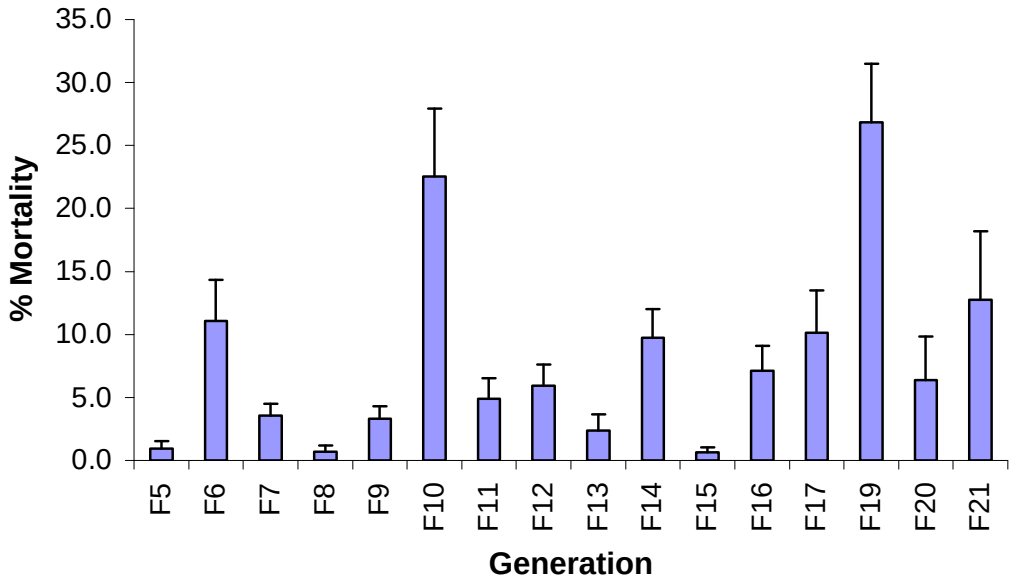
Cross	Number tested (n)	Mean percentage mortality observed	Percentage mortality expected (completely dominant)	Percentage mortality expected (completely recessive)
FUMOZ-R	222	9.7 ± 2.5	-	-
FANG	164	100 ± 0	-	-
B1 (F1 hybrid)	259	85.3 ± 5.0	-	-
B2 (F1 hybrid)	141	58.2 ± 4.7	-	-
H1 (Backcross to RR)	76	8.7 ± 5.4	9.7	50*
H3 (Backcross to RR)	56	2.8 ± 2.8	9.7	50*
H2 (Backcross to SS)	76	57.5 ± 6.6	50	100*
H4 (Backcross to SS)	136	49.8 ± 8.1	50	100*
B1F2 (F2 hybrid)	76	56.3 ± 11.0	25	75
B2F2 (F2 hybrid)	89	69.4 ± 14.0	25*	75

(*) Expected survival significantly different from observed mean survival at $p<0.05$ using 1-sample t tests.

3.4.6 Stability of resistance in the FUMOZ base colony

Figure 3.6 shows the mortality response of 16 generations of the baseline FUMOZ colony following exposure to 0.75% permethrin for 1 h. These results show that in the absence of insecticide selection, the frequency of permethrin resistance fluctuated between generations. However, resistance did not show any consistent trend over several generations without selection. Mortalities 24 h post exposure at the recommended WHO dosage at all except two generations were far below the WHO resistance level discriminator of 80% mortality using 0.75% permethrin for 1 h.

Figure 3.6: Mortalities 24 h post exposure of several generations of the unselected *An. funestus* FUMOZ base colony following exposure to 0.75% permethrin for 1 h.



3.5 DISCUSSION

Understanding the genetic basis of insecticide resistance may assist with developing strategies to manage mosquito resistance to insecticides. The mode of inheritance of permethrin resistance in southern African *An. funestus* was determined using a single dose of permethrin against F1 hybrids, the offspring of hybrid intercrosses (F2) and backcrosses to the resistant and susceptible parental strains. Crow (1957) recommended the procedure of backcrossing to the susceptible parent as a technique for studying the inheritance of insecticide resistance genes. The studies of Davidson (1958) on dieldrin resistance in *An. gambiae* was central to the understanding of resistance inheritance in the *An. gambiae* complex. Davidson (1958) crossed resistant and susceptible strains of *An. gambiae* and, by determining the proportional expression of resistance of the offspring hybrids of parental backcrosses concluded that inheritance of dieldrin resistance was monofactorial. The hybrids from reciprocal crosses of the resistant and susceptible strains were found to be intermediate in their resistance to dieldrin and gamma-BHC (Davidson, 1958).

The mortality response of heterozygous offspring (F1 resulting from reciprocal crosses between resistant and susceptible adults) to permethrin exposure were similar during the 60 minutes exposure period but showed significant differences 24 h post exposure. There was no indication of sex linkage with the resistance phenotype because the results indicate that both female and male parents were capable of transmitting the resistance character to F1 hybrids.

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Data on insecticide susceptibility of F1 progeny, as well as F2 progeny resulting from F1 backcrosses to susceptible and resistant parental colonies were consistent with an autosomal mode of inheritance in which the resistant genes presented as incompletely dominant. The results indicate a major factor, but it is possible that minor modifier gene(s) might also be involved. Monofactorial inheritance has been reported in previous studies of insecticide resistance: dieldrin resistance in *An. gambiae* and *An. sundaicus* (Davidson, 1958) and DDT resistance in *An. gambiae* (Haridi, 1972; Ranson *et al.*, 2000b).

The time knockdown/mortality response of the backcross progeny following exposure to permethrin showed that after 60 minutes the knockdown rates for H1 and H3 correlated best with the rate recorded for the resistant parental strain. However, the final mortalities 24 h post exposure recorded for the backcross progeny (H1 and H3) were lower than the knockdown rates observed after 60 minutes. More than half that were knocked down subsequently recovered. A similar effect was recorded in those progeny produced by backcrosses to the susceptible parents (H2 and H4) with slightly less than 50% of the knocked down mosquitoes recovering 24 h post exposure. Theoretically, a metabolic resistance mechanism such as that proposed for FUMOZ-R (Brooke *et al*., 2001) would produce a resistance phenotype based on the amount of detoxifying enzyme produced. This, in turn, would depend on various factors including resistance locus genotype (RR, RS, SS), relative body mass and other physiological factors. The varying response to a single discriminating dose of insecticide reflects the influence of these factors.

The final mortalities 24 h post exposure obtained for the F2 progeny resulting from F1 intercrosses deviated significantly from the 25% mortality expected under the assumption of a single, dominant controlling factor (Wilson *et al*., 1973). The result rather tended toward a recessive phenotypic expression where 75% mortality would be expected under the assumption of a single, recessive controlling factor. Generally, the results obtained from the crosses and backcrosses suggest that resistance is inherited autosomally in an incompletely dominant manner. These results are consistent with previous studies implicating monooxygenase based detoxification as the primary mode of resistance with the possible involvement of glutathione S-transferase (Brooke *et al*., 2001). These results are further supported by Wondji *et al*., (2007a) who chromosomally mapped a single quantitative trait locus (QTL) associated with pyrethroid resistance using the same FUMOZ-R and FANG strains. They found that a QTL which accounts for more than 60% of variance in susceptibility to permethrin is located within division 9 of chromosome arm 2R. This location coincides with a cluster of CYP6 p450 monooxygenases mapped by fluorescent in situ hybridisation, suggesting that resistance is mediated by one or more CYP6 p450 genes within this cluster. Interestingly, Cyp6P9 (the *An. funestus* ortholog of *An. gambiae* Cyp6P3) is found within this cluster and has been found to be overexpressed in the FUMOZ-R strain (Koekemoer, pers comm.).

The mode (polygenic or monogenic) of inheritance of insecticide resistance can influence the rate of evolution of resistance (Roush & McKenzie, 1987). Monogenic resistance is more likely to spread within field populations than is polygenic

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resistance (Roush & McKenzie, 1987). This is because a monogenically resistant individual that emigrates into a susceptible population is more likely to leave some resistant progeny (Roush & McKenzie, 1987). If resistance is monogenic, the rate of resistance development can be influenced by the dominance of resistance (i.e. the relative survival of RS heterozygotes relative to the homozygous parents under field exposure (Roush & McKenzie, 1987)). The degree of expression of dominance depends on the chemical and on the dose applied (Bouvier *et al*., 2001). If an insecticide deposit in the field is strong enough to kill heterozygotes, the resistance would be functionally recessive, but at doses insufficient to kill heterozygotes the resistance would be functionally dominant (Curtis *et al*., 1978). Bioassay data from this study indicate that permethrin resistance was incompletely dominant. However, care has to be taken in the extrapolation of laboratory data to field conditions because the concentration of insecticide that establishes relative dominance may differ between laboratory and field populations (May & Dobson, 1986; Roush & McKenzie, 1987). Another factor to consider is reduction in the efficacy of residual applications owing to the degradation of the insecticide in the natural environment (Roush & McKenzie, 1987). As a result of this the resistance level expressed in heterozygotes could remain high enough to allow survival (Bouvier *et al*., 2001).

The bioassay results for crosses of the resistant and susceptible strain of *An. funestus* studied here showed that the F1 progeny from the reciprocal crosses (RS) were less resistant than their resistance selected parents which were presumed to be homozygous (RR). In addition, bioassay results from FUMOZ base colony

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maintained through several generations showed that resistance was maintained in the colony in the absence of insecticide selection. Consequently, in the field, resistance may not dissipate in the absence of pyrethroid selection pressure because these data indicate that the resistance gene is stable and that there was no strong evidence for fitness cost associated with pyrethroid resistance (Okoye *et al.*, 2007).

Future work could be carried out by testing the different backcross progeny over a range of doses and plotting the dose/mortality graph to determine whether there is a plateau at 50% mortality. This would help discriminate the action of a single major gene from that of contributions of several genes of lesser effect.

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CHAPTER 4

SEQUENCE ANALYSIS OF DOMAIN II OF THE SODIUM CHANNEL GENE IN *AN. FUNESTUS*

4.1 INTRODUCTION

Pyrethroid resistance has developed in many arthropods including mosquitoes (Brown, 1986; WHO, 1992; Hemingway & Ranson, 2000). Voltage-gated sodium channels are the target for both pyrethroid insecticides and DDT (Liu *et al*., 2006). Mutations in the sodium channel gene have been associated with DDT/pyrethroid resistance in various arthropods (see Chapter 1). All the sodium channel mutations so far identified in mosquitoes are in domain II (Soderlund & Knipple, 2003; Hemingway *et al.*, 2004; Liu *et al.*, 2006). The aim of this chapter was to further characterize pyrethroid resistance in southern African *An*. *funestus* by comparing the IIS5 – IIS6 regions of the sodium channel gene between pyrethroid susceptible and resistant laboratory strains. In addition, the study aimed to compare introns and coding regions of the sodium channel gene between the two strains. To achieve this, genomic DNA and cDNA was synthesized, PCR amplified, cloned and sequenced in both resistant and susceptible strains of *An*. *funestus*.

4.2 MATERIALS AND METHODS

The detailed methodology used to sequence, analyze and compare the S5 - S6 transmembrane segment of the domain II region of the sodium channel from pyrethroid resistant and susceptible strains of *An. funestus* is presented in Appendix C.

4.2.1 Laboratory strains of *An. funestus*

The two *An. funestus* mosquito strains used in this study are FUMOZ-R (a pyrethroid resistant strain from southern Mozambique) and FANG (a pyrethroid susceptible strain originating from southern Angola). Details of these strains and their rearing conditions are given in Chapter 2.

4.2.2 PCR Optimization

Initial PCR optimization was carried out using primers Agd1, Agd2, Agd3, Agd4, D1, Dipd1, Dg1 and Dg2 (Martinez-Torres *et al.*, 1998). Different concentrations of $MgCl_2$ (between 0.5 mM and 3 mM) and annealing temperatures (between 40°C and 60°C) were used and the thermocycling conditions were varied. As a starting point, an annealing temperature 5°C below the melting temperature (Tm) was used. It was then adjusted to improve specificity and yield of PCR products. PCR products were

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electrophoresed on 1.5% (w/v) agarose gels in 1X TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8) containing 10 mg/ml ethidium bromide (Sigma, cat no. E-1510). Electrophoresis was performed at a constant voltage of 100 V in 1 X TAE buffer. Size markers were loaded onto the agarose gels and PCR products were visualized using a UV-illuminator (Syngene G:BOX, Cambridge, UK).

No amplification was achieved using the above primers on *An. funestus*. As a result new primers were designed as described below. The same optimization procedure as described previously was carried out for the new primer sets. The optimal conditions obtained using the new primer sets were used for subsequent PCR reactions.

4.2.3 Primer design

PCR specificity and efficiency was improved by designing the primers such that complementarities between primer sequences were avoided (Strachan & Read, 1999). Further, a run of three or more guanine (G) or cytosine (C) bases at the 3' -terminal sequence of each primer was avoided in order to prevent non-specific annealing (Strachan & Read, 1999). Primer length was 20 bp as a primer length of 20 bases is considered optimal for most PCR applications (Strachan & Read, 1999).

The sequence of a partial cDNA encoding this particular region of the voltage-gated sodium channel of *An. gambiae* (accession no. Y13592) was downloaded from the

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GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/) and used to design primers for amplification of the homologous region in *An. funestus*. Two introns have been identified in the S5 - S6 transmembrane segments of the sodium channel gene of various insects (Loughney *et al.*, 1989; Martinez-Torres *et al*., 1998; Martinez-Torres *et al*., 1999). Primers were designed to span the entire region and individual primers were also designed to amplify each of the two introns. PrimerSelect in the DNASTAR suite of programs and Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) were used for primer design. Three sets of primers were designed. Primers N1 and N2 flank intron 1, primers N3 and N4 flank intron 2 while primers N1 and N4 flank the whole region of interest. The schematic representations of each of the primers are given in Figure 4.1 and the sequences of all the primers used in this study are presented in Table 4.1.

The primers designed were different from those used by Wondji *et al.* (2007b). The primers used in this study amplified ~ 1424 bp of PCR product compared to the 1344 bp obtained by Wondji *et al.* (2007b) (accession no. DQ534436.1), giving an additional ~ 86 bp (5' -terminal (1 – 42 bp) and 3' -terminal (1380 – 1424 bp) of the sequence obtained from this study).

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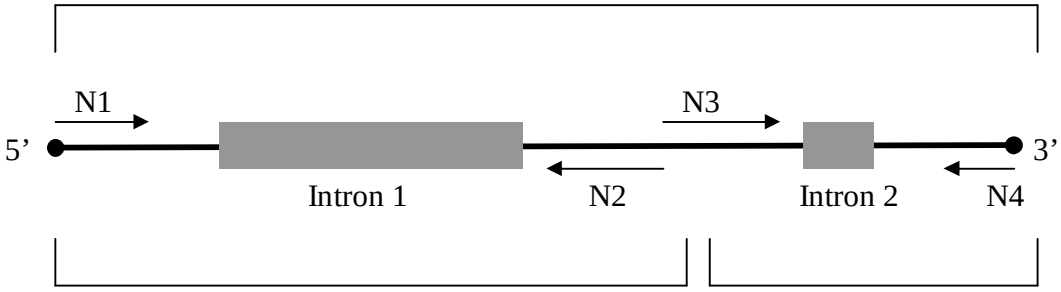
Table 4.1: Sequences of primers used in this study. N1, N2, N3 and N4 are primers designed to amplify the IIS5 – IIS6 region of the sodium channel gene in *An. funestus*. Universal primers T7, SP6, M13 forward (-40) and M13 reverse were used in the sequencing of the plasmids. Primers UV, FUN, VAN, RIV, PAR and LEES (Koekemoer *et al.*, 2002) were used for species identification.

Primer name		**TM(°C)**
N1	AAC GAT GGG TGC GTT AGG TA	60.4
N2	CGG AAC ACA ATC ATG AAG GA	58.35
N3	GTG CTA TGC GGA GAA TGG AT	60.4
N4	GTT GGT GCA GAC AAG GAT GA	60.4
T7	GTA ATA CGA CTC ACT ATA G	45
SP6	CAT TTA GGT GAC ACT ATA G	45
M13 forward (-40)	GTT TTC CCA GTC ACG AC	47
M13 reverse	AAC AGC TAT GAC CAT G	41
UV	TGT GAA CTG CAG GAC ACA T	55.34
FUN	GCA TCG ATG GGT TAA TCA TG	52.4
VAN	TGT CGA CTT GGT AGC CGA AC	58
RIV	CAA GCC GTT CGA CCC TGA TT	58.8
PAR	TGC GGT CCC AAG CTA GGT TC	60.5
LEES	TAC ACG GGC GCC ATG TAG TT	60.2

Figure 4.1: A schematic representation of the primers used in this experiment. The positions of the introns are shown in blocks ( ).

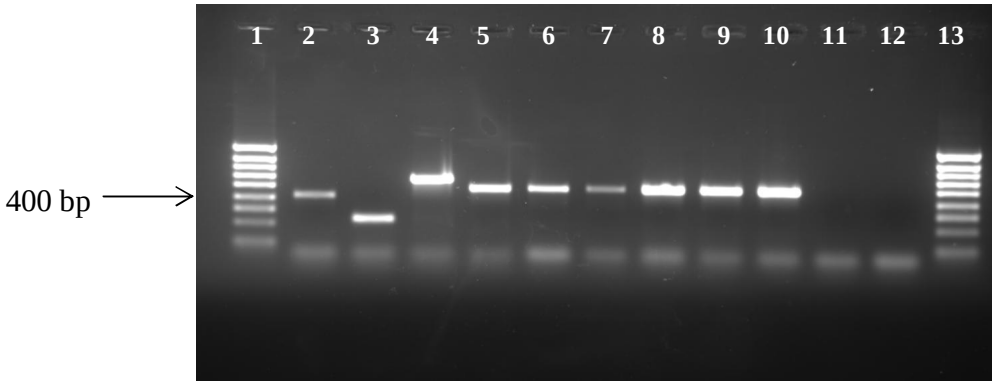


4.2.5 Sequencing of the IIS5 - IIS6 domain of the sodium channel gene using genomic DNA

Genomic DNA was extracted individually from at least six mosquitoes from each of the FUMOZ-R and FANG strains according to the method of Collins *et al.* (1987) as described in Appendix C. Fifty to 100 nanograms of DNA was used per PCR reaction. Although the *An. funestus* samples used for this study were drawn from colony material the species identity of all specimens was re-confirmed by using the species-specific PCR assay of Koekemoer *et al.* (2002). Details of the PCR conditions are presented in Appendix C and the sequences of the primers used (UV, FUN, VAN, RIV, PAR and LEES) are given in Table 4.1. PCR products were electrophoresed on a 1.5% (w/v) agarose gel in 1 X TAE buffer (containing 10 mg/ml

ethidium bromide (Sigma, cat no. E-1510) and visualized using a UV-illuminator (Syngene G:BOX, Cambridge, UK) (Figure 4.2).

Figure 4.2: Species-specific identification of *An. funestus*. Lanes 1 and 13 = molecular weight marker (Hyperladder IV, Bioline, UK. cat no. BIO-33029), lane 2 = positive control (*An. rivulorum* = 411 bp); lane 3 = positive control (*An. leesoni* = 146 bp), lane 4 = positive control (*An. vaneedeni* = 587 bp), lane 5 - 7 = FANG (*An. funestus* = 505 bp), lane 8 - 10 = FUMOZ-R (*An. funestus* = 505 bp), lane 11 - 12 = negative control.



The introns were sequenced directly using PCR products. The entire region of interest was PCR amplified using primers N1, N2, N3 and N4; intron 1 was amplified using primers N1 and N2 and intron 2 was amplified using primers N3 and N4 (Table 4.1). The internal primers N2 and N3 were used so as to ensure that the entire sequence was obtained given that the product size of the region is ~1400 bp. For each reaction,

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10 X PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl), 3.3 µM of each primer and 2 mM $MgCl_2$ and one unit of Takara *Taq* polymerase (Takara Bio Inc. Japan, code R001AM) was used in a 25 µl total PCR volume. Prior to amplification, 50 – 100 ng of DNA template was added per reaction. Amplification was performed at an initial denaturation of 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds with a final extension step of 72°C for 5 minutes. Five µl of the PCR product was electrophoresed on a 1.5% (w/v) agarose gel in 1 X TAE buffer containing 10 mg/ml ethidium bromide (Sigma, cat no. E-1510) and visualised using a UV-illuminator (Syngene G:Box, Cambridge, UK). The remaining PCR product was stored at -20°C until needed for sequencing.

4.2.6 Sequencing of the IIS5 - IIS6 domains of the sodium channel gene using mRNA

Total RNA was extracted using TRI Reagent® (Sigma, USA, T9424-100ml) according to the manufacturers' protocol (see Appendix C). cDNA synthesis was carried out using the ImProm-II™ Reverse Transcription System (Promega, USA, cat no. A3800) (see Appendix C). The cDNA was quantified using the GENE QUANT *pro* (Amersham Biosciences, 89761) or the NANODROP (ND 1000) spectrometer and was then used as a template for PCR using N1 and N4 primers. The PCR conditions were the same as previously described for genomic DNA (section 4.2.5). Due to difficulty in PCR direct sequencing from cDNA, the PCR products were

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purified and cloned. One hundred µl of PCR product was gel purified using a QlAquick Gel Extraction kit (Qiagen, USA, cat no. 28704) (see Appendix C). Purified PCR products were cloned using the Qiagen PCR cloning kit (Qiagen, USA, cat no. 231124) (see Appendix C). White/blue colonies were screened for correct insert by picking isolated individual clones and carrying out PCR using the T7 and SP6 primers (Table 4.1). Plasmids were purified using the Qiagen plasmid mini kit (described in Appendix C) and used for sequencing.

4.2.7 Sequencing and sequence analysis

Sequencing reactions were carried out using the BigDye Version 3.1 terminator cycle sequencing kit (Applied Biosystems, USA, part no. 4337455) with the samples loaded into a SpectruMedix SCE2410 genetic analyser (SpectruMedix LLC, Pennsylvania, USA) using protocols recommended by the manufacturer. The sample preparation method is described in Appendix C. Alternatively, purified plasmids or PCR products were sent to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa, for sequencing. Genomic DNA was sequenced in both directions using the same primers used for PCR (N1, N2, N3 and N4). cDNAs were sequenced on both strands using primers SP6 and T7 or M13 forward (-40) and M13 reverse primers. Two cDNA samples and six genomic DNA samples were sequenced for each of FUMOZ-R and FANG and each sample was sequenced three times. Sequences were aligned using MegAlign software and analyzed using the DNASTAR® Lasergene software suite (DNASTAR Inc.).

4.2.8 Phylogenetic analysis

The relationship between the introns and coding regions of *An. funestus* and *An. gambiae* were examined by phlyogenetic analysis using MegAlign in the DNASTAR Lasergene software suite (DNASTAR Inc.).

4.3 RESULTS

Partial cDNA and genomic DNA sequencing was carried out on two laboratory colonies of *An. funestus* (FUMOZ-R and FANG). Basic Local Alignment Search Tool (BLAST) analysis of the sequences against the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/) confirmed that the sodium channel fragment had been amplified. The DNA sequences obtained from this study have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) (Table 4.2).

Table 4.2: GenBank accession numbers of genomic and mRNA sequences of FANG and FUMOZ-R.

Sequence name	**GenBank accession number**
Genomic sequence of FANG	DQ399296
mRNA sequence of FANG	DQ399297
Genomic sequence of FUMOZ-R	DQ399298
mRNA sequence of FUMOZ-R	DQ399299

4.3.1 Intron sequences

The primer sets (N1, N2, N3 and N4) were used with genomic DNA as templates to obtain the nucleotide sequence of the region of interest. Primers N1 and N2 (Figure 4.3) gave a band size of 1183 bp and 1185 bp for the FANG and FUMOZ-R strains respectively. Primers N3 and N4 (Figure 4.4) yielded a band size of 238 bp for both strains while primers N1 and N4 (Figure 4.5) yielded a band size of 1422 bp and 1424 bp for the FANG and FUMOZ-R strains respectively. Overall, a total of 1,422 bp (FANG) and 1424 bp (FUMOZ-R) was sequenced consisting of 351 bp of coding region (see section 4.3.2) for both strains and an intronic region of 1071 bp for FANG and 1073 bp for FUMOZ-R.

Figure 4.3: *Anopheles funestus* PCR products obtained using NI and N2 primers electrophoresed on a 1.5% agarose gel. Lane 1 = molecular weight marker (Hyperladder 1, Bioline, UK. cat no. BIO-33025), lane 2 = FUMOZ-R, lane 3 = negative control.

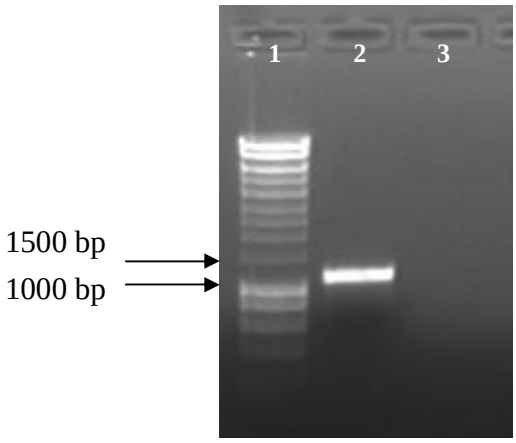


Figure 4.4: *Anopheles funestus* PCR products obtained using N3 and N4 primers electrophoresed on a 1.5% agarose gel. Lane 1 = molecular weight marker (Hyperladder 1, Bioline, UK. cat no. BIO-33025), lanes 2 - 4 = FANG, lanes 5 - 7 = FUMOZ-R, lane 8 = negative control.

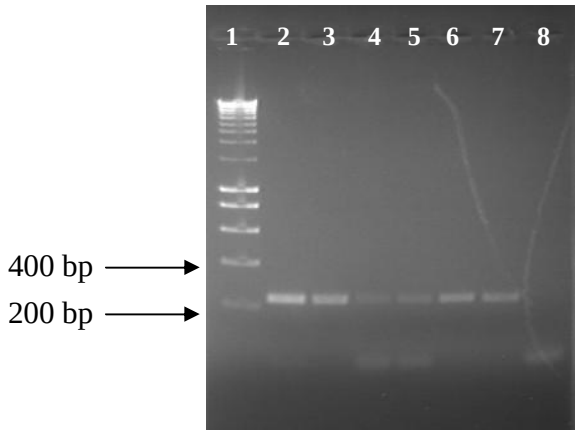
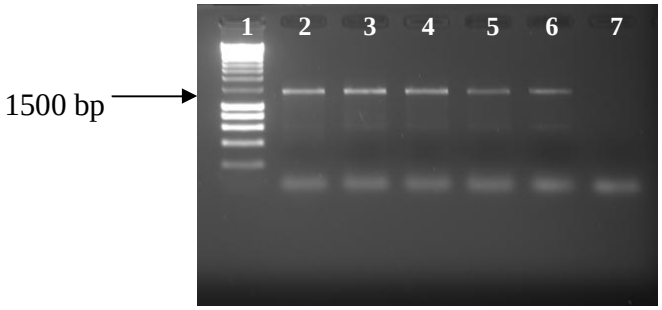


Figure 4.5: *Anopheles funestus* PCR products obtained using NI and N4 primers electrophoresed on a 1.5% agarose gel. Lane 1 = molecular weight marker (Hyperladder 1, Bioline, UK. cat no. BIO-33025), lanes 2 - 4 = FANG, lanes 5 - 6 = FUMOZ-R, lane 7 = negative control.



Two introns are known to occur in the genomic DNA of this segment in various insect species ((Loughney *et al.*, 1989; Martinez-Torres *et al.*, 1998; Martinez-Torres

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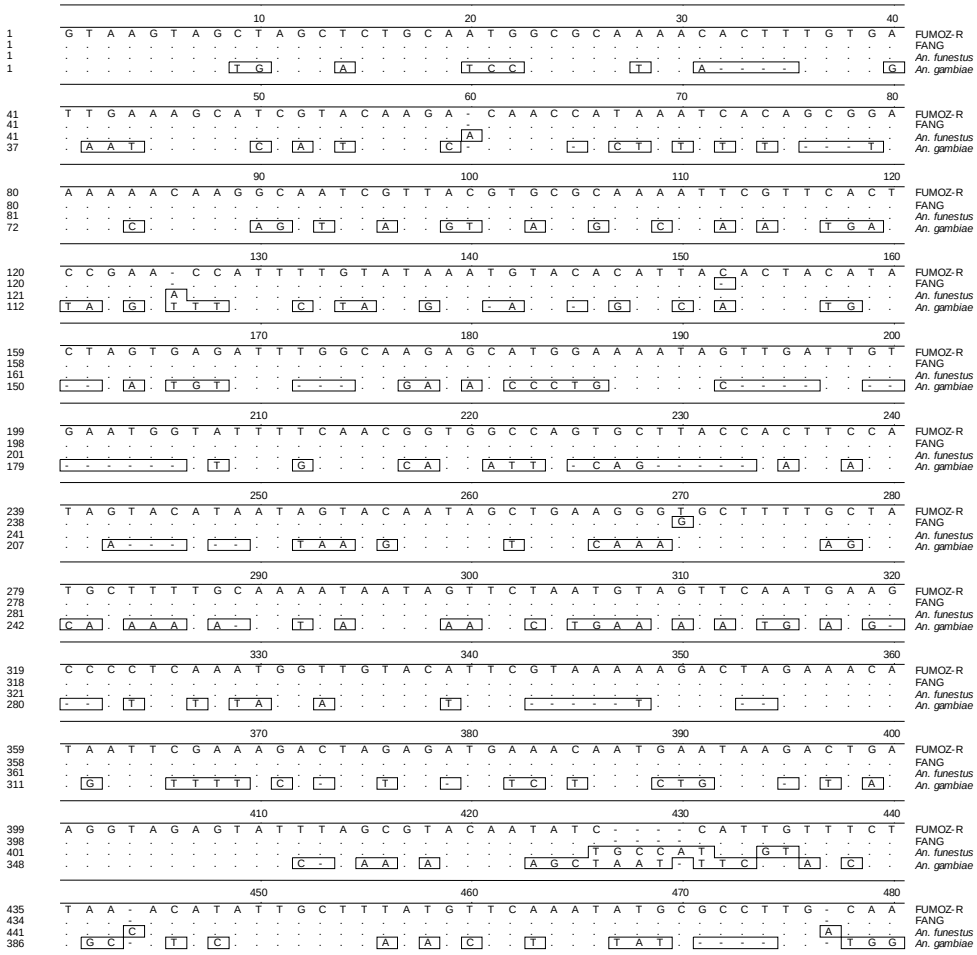
et al., 1999). To verify the position, sequence and length of these introns, cDNA was synthesized from both susceptible and resistant strains and amplified using the primers N1 and N4. The fragment obtained was aligned against the genomic sequences and this revealed the presence of two introns: one upstream (intron 1) and one downstream (intron 2) of the supposedly *kdr* mutation. The intron-exon boundaries of the partial sequences conformed to the canonical GT-AG rule (Mount, 1982).

Further analysis revealed a 1003 bp and 1005 bp intron 1 for the susceptible strain and resistant strains respectively (Figure 4.6). Aligning the two sequences showed that there was 99.4% identity between the two strains. The intron 1 of FUMOZ-R and FANG had a 59.6% and 59.5% identity respectively with the 920 bp intron 1 of *An. gambiae* (obtained from Weill *et al.*, 2000, no accession number given) (Figure 4.6). The alignment also showed a 99% sequence identity between the intron 1 of FUMOZ-R and FANG respectively with the intron 1 obtained from the consensus sequence by Wondji *et al.* (2007b) (accession no. DQ534436.1).

Intron 2 was 68 bp in length for both FUMOZ-R and FANG strains and no polymorphisms were detected. This intron was larger than the 57 bp intron described in *An. gambiae* (Martinez-Torres *et al.*, 1998) with only 66.7% sequence identity between the two (Figure 4.7). A sequence identity of 97.1% was found between intron 2 of FUMOZ-R and the intron 2 obtained from the *An. funestus* consensus

sequence of Wondji *et al.* (2007b). Nucleotide sequences for FANG are not shown in Figure 4.7 because the sequences for FANG and FUMOZ-R are identical.

Figure 4.6: Alignment of the nucleotide sequences of intron 1 of the IIS5 - IIS6 region of the voltage-sensitive sodium channel in pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains of *An. funestus*, *An. funestus* consensus sequence (obtained from Wondji *et al.*, 2007b) and *An. gambiae* (obtained from Weill *et al.*, 2000). Identical positions to FUMOZ-R are indicated by a dot (.) and differences are indicated by a box.



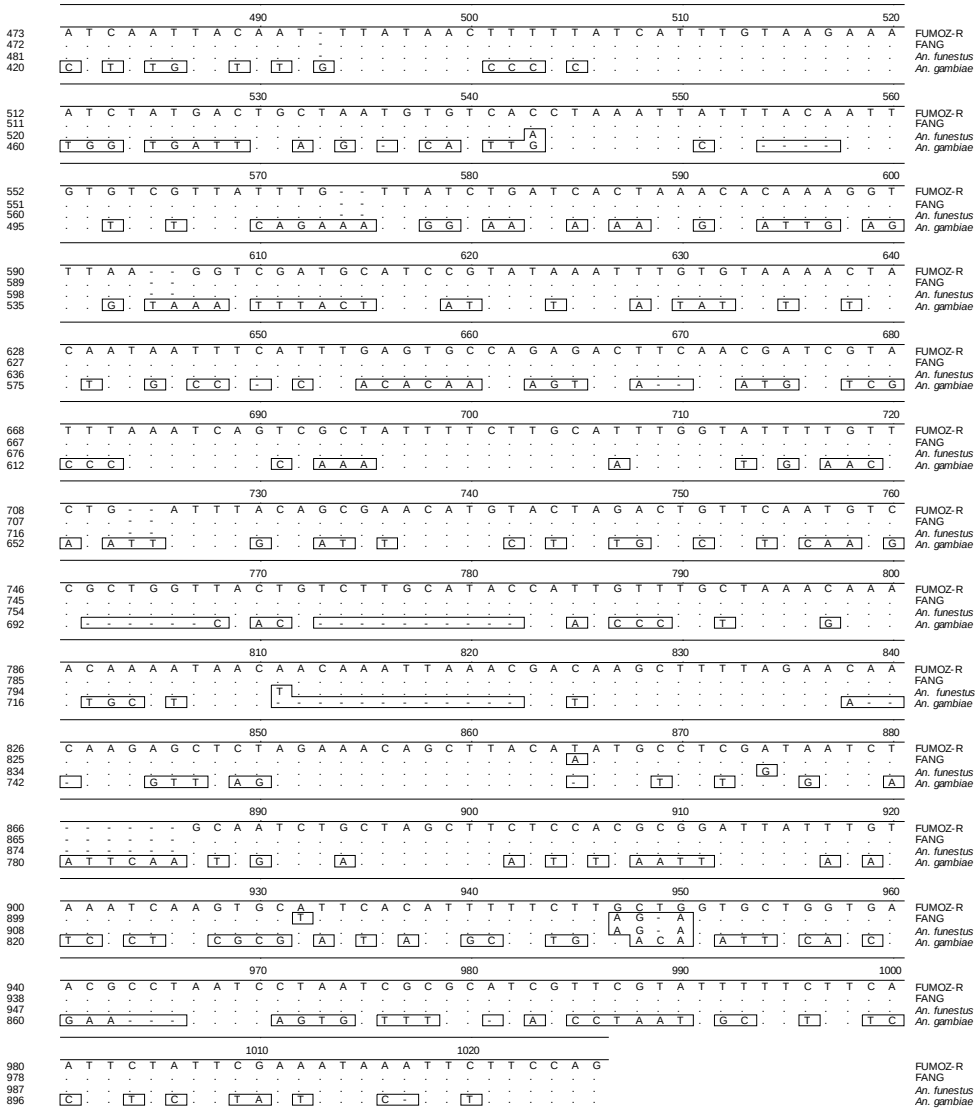
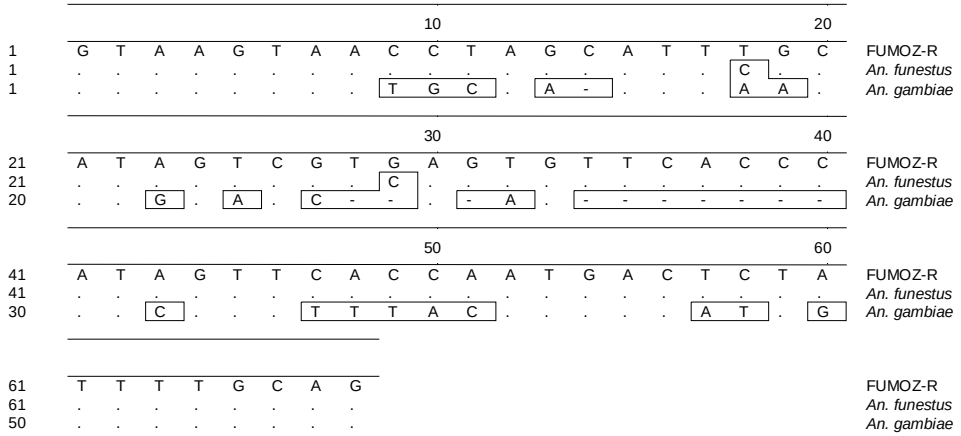


Figure 4.7: Alignment of the nucleotide sequences of intron 2 of the IIS5 - IIS6 region of the voltage-sensitive sodium channel in a pyrethroid resistant strain of *An. funestus* (FUMOZ-R), *An. funestus* consensus sequence (obtained from Wondji *et al.*, 2007b) and *An. gambiae* (obtained from Martinez-Torres *et al.*, 1998). Identical positions to FUMOZ-R are indicated by a dot (.) and differences are indicated by a box.



4.3.1.1 Phylogenetic analysis of intron sequences

Molecular phylogenetic analysis was carried out in order to determine the phylogenetic relationship between the intron sequences of FUMOZ-R, FANG and *An. gambiae*. The sequence of Wondji *et al.* (2007b) was excluded from the phylogenetic analysis since it was the consensus of three different *An. funestus* populations (FANG, FUMOZ-R and wild *An. funestus* populations from Malawi). Figures 4.8 and 4.9 show the phylogenetic relationship between the intron sequences

of FUMOZ-R, FANG and *An. gambiae* (Weill *et al.*, 2000) and confirms that the FUMOZ-R and FANG strains are more closely related to each other than either of them is to *An. gambiae* as is expected.

Figure 4.8: Phylogenetic relationship between intron 1 sequences of the IIS5 - IIS6 region of the voltage-sensitive sodium channel in pyrethroid resistant (FUMOZ-R), pyrethroid susceptible (FANG) *An. funestus* strains and *An. gambiae* (obtained from Weill *et al.*, 2000).

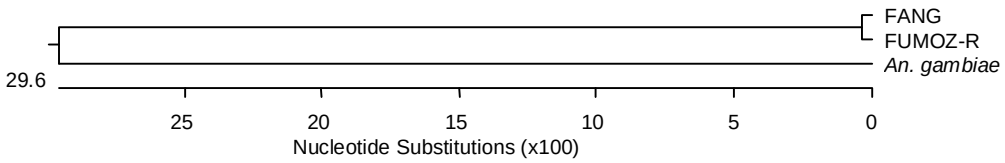
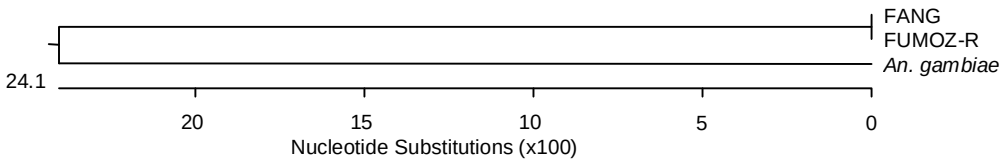


Figure 4.9: Phylogenetic relationship between intron 2 sequence of the IIS5 - IIS6 region of the voltage-sensitive sodium channel in pyrethroid resistant (FUMOZ-R), pyrethroid susceptible (FANG) *An. funestus* strains and *An. gambiae* (obtained from Martinez-Torres *et al.*, 1998).



4.3.2 mRNA sequences

PCR amplification of the *An. funestus* sodium channel gene using mRNA template yielded a 351 bp fragment (Figure 4.10) and this was cloned and sequenced. BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) analysis of both strands of the sequences revealed that the sodium channel fragment had been amplified. Tables 4.3 and 4.4 show a summary of BLAST results obtained as well as the percentage identity between FUMOZ-R and FANG sequences to those of other insects.

Figure 4.10: PCR products obtained using NI and N4 primers with cDNA as template and electrophoresed on a 1.5% agarose gel. Lane 1 = molecular weight marker (Hyperladder 1, Bioline, UK. cat no. BIO-33025), lanes 2 = FUMOZ-R, lane 3 = FANG, lane 4 = negative control.

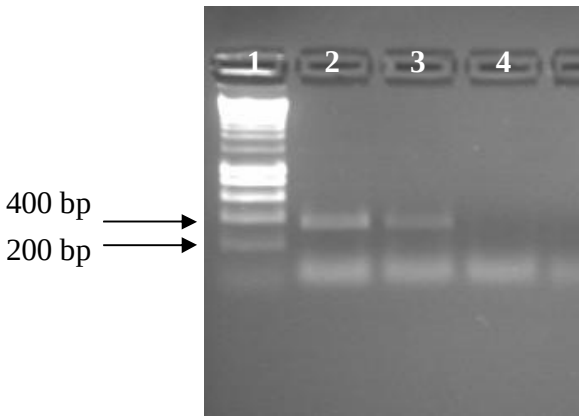


Table 4.3: BLAST results of FUMOZ-R mRNA sequences against the NCBI non-redundant database.

Accession number	Description	Query coverage	E value	Identity
DQ534436.1	*An. funestus*	70%	2e-89	98%
DQ334052.1	*An. sinensis*	43%	3e-33	96%
DQ333331.1	*An. subpictus*	15%	6e-15	96%
AY422491.1	*An. stephensi*	26%	5e-31	94%
XM_001231102.1	*An. gambiae* str. PEST ENSANGP00000032062	100%	1e-141	92%
Y13592.1	*An. gambiae*	100%	1e-141	92%
DQ408540.1	*C. p. quinquefasciatus*	90%	6e-95	86%
DQ538356.1	*Ae. albopictus*	99%	3e-98	85%
AY663385.1	*Ae. aegypti*	99%	3e-98	85%

Table 4.4: BLAST results of FANG mRNA sequences against the NCBI non-redundant database.

Accession number	Description	Query coverage	E value	Identity
DQ534436.1	*An. funestus*	69%	9e-88	100%
DQ333331.1	*An. subpictus*	15%	1e-16	98%
DQ334052.1	*An. sinensis*	43%	3e-33	96%
AY422491.1	*An. stephensi*	26%	5e-31	94%
XM_001231102.1	*An. gambiae* str. PEST ENSANGP00000032062	100%	1e-141	92%
Y13592.1	*An. gambiae*	100%	1e-141	92%
DQ538356.1	*Ae. albopictus*	99%	6e-100	86%
AY663385.1	*Ae. aegypti*	99%	6e-100	86%
DQ408540.1	*C. p. quinquefasciatus*	94%	1e-96	86%

A comparison of the two aligned nucleotide mRNA sequences obtained for the two strains revealed a 99.4% sequence identity between them with only two nucleotide

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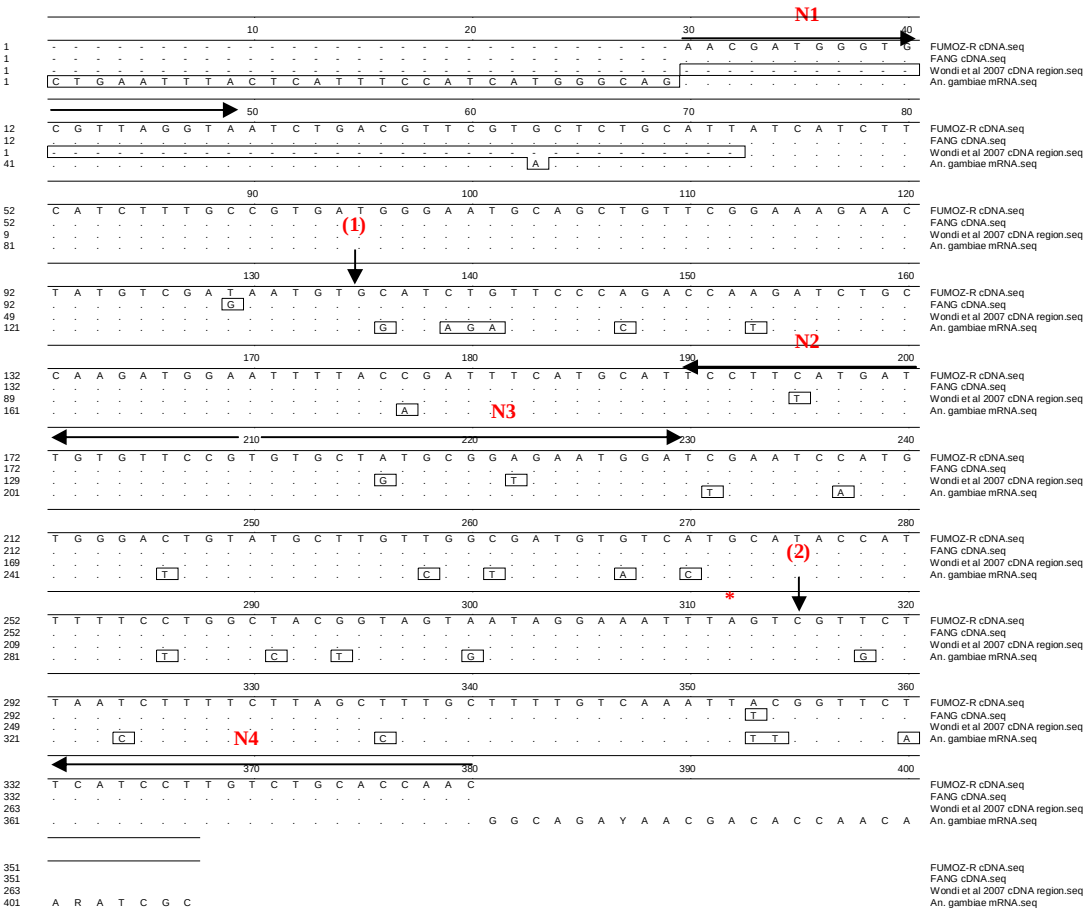
differences. The nucleotide differences were at positions 100 (guanine (G) in FANG compared to thymine (T) in FUMOZ-R) and 324 (thymine (T) in FANG compared to an adenine (A) in FUMOZ-R) (based on nucleotide position numbering of FUMOZ-R) (Figure 4.11). Comparison of the resultant 351 bp mRNA nucleotide sequence of the IIS5 - IIS6 region of pyrethroid resistant strains of *An. funestus* and *An. gambiae* (accession no. Y13592) showed a 92.9% identities between the two (Figure 4.11). The *kdr* mutation (mutation of an adenine (A) to thymine (T) at the *kdr* mutation position (indicated in Figure 4.11)) which had been reported in other insects including *An. gambiae* (Martinez-Torres *et al.*, 1998), *An. arabiensis* (Verhaeghen *et al.*, 2006; Matambo *et al.*, 2007) and *An. sacharovi* (Lüleyap *et al.*, 2002) was absent in FUMOZ-R.

Figure 4.11: Alignment of the mRNA sequence of the IIS5 - IIS6 region of the voltage-sensitive sodium channel gene in pyrethroid resistant (FUMOZ-R) and susceptible (FANG) *An. funestus* strains, *An. funestus* consensus sequence (obtained from Wondji *et al.*, 2007b) and *An. gambiae* (accession no. Y13592). Identical positions to FUMOZ-R are indicated by a dot (.) and differences are indicated by a box. (1) indicates intron 1 while (2) indicates intron 2. The supposedly *kdr* mutation position is indicated below *. Primers (N1, N2, N3 and N4) position are shown.



The deduced amino acid sequences of FANG and FUMOZ-R were 98.3% identical (Table 4.5 and Figure 4.12). The deduced amino acid sequence revealed two amino acid differences between FUMOZ-R and FANG at positions 32 and 107 (based on amino acid position numbering of FUMOZ-R) (Figure 4.12). At position 32 of the deduced amino acid alignment, a polar (negatively charged) hydrophilic glutamic acid residue (GAG) was present in FANG while a polar (uncharged) hydrophilic asparagine (GAT) was present in FUMOZ-R. At position 107 of the deduced amino acid alignment, a nonpolar (hydrophobic) phenylalanine residue (TTC) was present in FANG while a nonpolar (hydrophobic) tyrosine (TAC) was present in FUMOZ-R.

Table 4.5: Percentage sequence identity between the mRNA sequence of FUMOZ-R, FANG, *An. gambiae* (accession no. Y13592), consensus sequence of *An. funestus* (obtained from Wondji *et al.*, 2007b), the housefly *Musca domestica* (accession no. X96668) and *D. melanogaster* (accession no. M24285).

Species/strain	FUMOZ-R	FANG	*An. funestus*	*An. gambiae*	*M. domestica*	*D. melanogaster*
FUMOZ-R	-	98.3	96.6	96.5	91.3	93.0
FANG		-	95.5	96.5	91.3	93.0
An. funestus			-	93.2	86.4	88.6
An. gambiae				-	94.8	95.7
M. domestica					-	96.5

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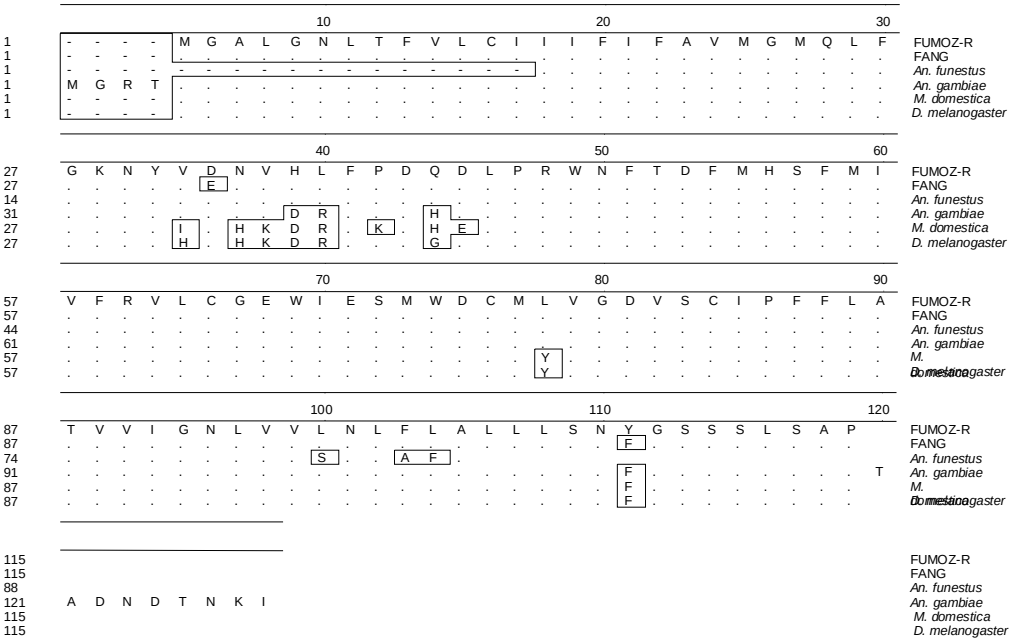
Figure 4.12 shows a comparison of the deduced amino acid sequence of the sodium channel gene of FANG and FUMOZ-R with that of the wild populations of *An. gambiae* (accession no. Y13592), consensus sequence of *An*. *funestus* (accession no. DQ534436.1), the housefly *Musca domestica* (accession no. X96668) and *Drosophila melanogaster* (accession no. M24285). The deduced amino acid alignment revealed that the FUMOZ-R sequence had a 96.6%, 96.5%, 91.3% and 93% identity to the consensus sequence of *An. funestus*, *An. gambiae*, *M. domestica* and *D. melanogaster* sodium channel genes respectively (Table 4.5).

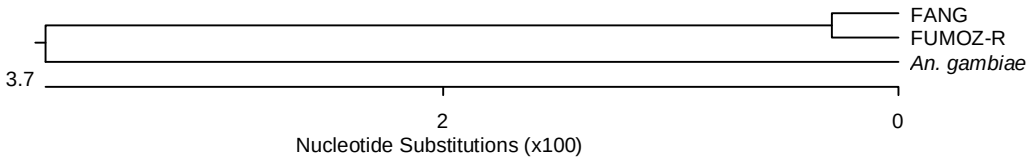
Figure 4.12: Alignment of the deduced amino acid sequences of the sodium channel gene in pyrethroid resistant (FUMOZ-R) and susceptible (FANG) strains of *An. funestus* and *An. funestus* consensus sequence (obtained from Wondji et al., 2007b), *An. gambiae* (accession no. Y13592), *M. domestica* (accession no. X96668) and *D. melanogaster* (accession no. M24285). Identical positions to FUMOZ-R are indicated by a dot (.) and differences are indicated by a box.



4.3.2.1 Phylogenetic analysis of mRNA sequences

Molecular phylogenetic analysis was carried out in order to determine the phylogenetic relationship between the mRNA sequences of FUMOZ-R, FANG and *An. gambiae* (accession no. Y13592) (Figure 4.13). The phylogenetic tree confirms that the FUMOZ-R and FANG strains are more closely related to each other than either of them is to *An. gambiae* as is expected.

Figure 4.13: Phylogenetic relationship between the mRNA sequence of the IIS5 - IIS6 region of the voltage-sensitive sodium channel in pyrethroid resistant (FUMOZ-R) and susceptible (FANG) *An. funestus* strains and *An. gambiae*.



4.4 DISCUSSION

It is important to note that the study carried out in this Chapter was done without prior knowledge that a group at the Liverpool School of Tropical Medicine were working on the same gene using the same laboratory colony used in this study. Their results were published (Wondji *et al.*, 2007b) before the submission of this thesis. There are substantive differences in methodology and data analysis between the two

studies which are highlighted as follows. Firstly, Wondji *et al.* (2007b) used only genomic DNA while both genomic DNA and mRNA were synthesized and used in this study in order to identify the coding and the intronic regions. Secondly, the region they amplified was ~ 86 bp smaller than the region sequenced in this study and they did not determine the phylogenetic relationship between FUMOZ-R and FANG. Finally, Wondji *et al.* (2007b) combined sequences of FUMOZ-R, FANG and Malawian field populations of *An. funestus*. They deposited one sequence (DQ534436) comprising of all three strains in GenBank, with no indication as to whether sequence differences were observed between the respective strains. The sequences obtained in the present study were deposited in GenBank separately as either mRNA or genomic sequences for FANG and FUMOZ-R respectively (Table 4.2).

Partial sequencing of the domain II region of the sodium channel gene was carried out on FUMOZ-R and FANG in order to determine whether there were any mutations associated with pyrethroid resistance. The sequence of the introns was also analysed in the two *An. funestus* strains in order to establish whether the introns would be a good tool with which to compare them given that they originate from different geographical locations. According to the hypothesis that introns are generally under low selection pressure, more differences may be expected in larger introns than in smaller introns of closely related species. Although coding and non-coding DNA are equally susceptible to mutation, the overall mutation rate in coding DNA is normally lower than that in non-coding DNA (Strachan & Read, 1999). This is because

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mutations (such as deletions/insertions of one or several nucleotides) might cause a shift in the translational reading frame (frameshift mutation), introducing a premature termination codon and causing loss of gene product or change of key coding function (Strachan & Read, 1999).

Comparison of the mRNA sequences of the sodium channel gene from the resistant and susceptible strains showed that they were extremely similar at the nucleotide deduced amino acid levels with only a few differences. The close genetic relationship in the sodium channel gene of *An. funestus* and *An. gambiae* suggests that the function of the sodium channel gene is conserved in the two species. Additional support for this conclusion was provided by the 92.9% identity of the resultant 351 bp mRNA nucleotide sequence and the 96.5% amino acid sequence identity recorded between the two species. This is higher than the 70% nucleotide and 50% amino acid at <300 bp mRNA sequence identity recorded by Lüleyap *et al.* (2002) when they compared *An. sacharovi* and *An. gambiae* voltage-gated sodium channel gene sequences. Enayati *et al.* (2003) reported an 88% identity of 237 bp genomic DNA between *An. stephensi* and the equivalent fragment in the *An. gambiae* sodium channel gene.

Two introns are known to exist in the domain II region of the sodium channel gene at conserved positions in the *Drosophila* (Loughney *et al.*, 1989), *M. domestica* (Martinez-Torres *et al.*, 1999) and *An. gambiae* (Martinez-Torres *et al.*, 1998). The positions of the introns in *An. funestus* were found to be conserved with respect to the

sequences of these insects but the sequences were not identical. The percentage of variable sites between FUMOZ-R and FANG was low (0.6%). The 1003 and 1005 bp of intron 1 for the resistant and susceptible strains of *An. funestus* respectively was greater than the 920 bp reported in *An. gambiae* by Weill *et al.* (2000). The percentage of variable sites within intron 1 in FUMOZ-R/FANG compared with *An. gambiae* was ~ 40%. Low polymorphism has been found in intron 1 of *An. gambiae* populations (Weill *et al.*, 2000; Gentile *et al.*, 2004; Pinto *et al.*, 2006). Gentile *et al.* (2004) recorded 2.4% variability in intron 1 of *An. gambiae*, while 7.5 – 18.6% variability has been found in other intronic regions of other *An. gambiae* populations (Gentile *et al.*, 2001). No polymorphism was detected in intron 2 between the resistant and susceptible strains of *An. funestus*. The percentage of variable sites in intron 2 between either FUMOZ-R or FANG and *An. gambiae* was 33.3%. No polymorphism in intron 2 has been recorded between *An. gambiae* populations (Martinez-Torres *et al.*, 1998; Weill *et al.*, 2000; Gentile *et al.*, 2004; Pinto *et al.*, 2006). The 68 bp intron 2 in *An. funestus* was 11 bp greater in size than the 57 bp reported in *An. gambiae* (Martinez-Torres *et al.*, 1998). However, it was smaller than those reported in other species; ~ 130 bp in other dipterans (unpublished results in Martinez-Torres *et al.*, 1998) and 106 bp in *An. stephensi* (Enayati *et al.*, 2003).

The voltage-gated sodium channel is the target site for both DDT and pyrethroid insecticides (Williamson *et al.*, 1996; Martinez-Torres *et al.*, 1998, 1999). It has been suggested that the *kdr*/super *kdr* mutations may be functionally important and may induce a conformational change in segment 6 thus affecting the binding of the

pyrethroid and DDT insecticide to the sodium channel (Williamson *et al.*, 1996; Park & Taylor, 1997; Martinez-Torres *et al.*, 1999; Ranson *et al.*, 2000a). Sequence analysis of this region in the pyrethroid resistant strain of *An. funestus* shows that *kdr*-type mutations were absent. This suggests that target site insensitivity is not associated with pyrethroid resistance in FUMOZ-R. The absence of *kdr*-type mutations reinforces other evidence showing that pyrethroid resistance in the FUMOZ-R strain is metabolic, essentially based on the overproduction of monooxygenases with the possible involvement of GSTs (Brooke *et al.*, 2001; Wondji *et al.*, 2007a).

The relatively low level of polymorphism found in the sodium channel gene sequence of the FUMOZ-R and FANG strains suggests that the two are not genetically different (in this region of the genome) although the wild populations from which they were derived are geographically separated. Further evidence in support of this conclusion comes from the following studies. Analyses of *Msp I* digests of a 400 bp domain 3 (D3) fragment of *An. funestus* from samples collected in west, central, east and southern Africa show clear evidence of population structuring (Garros *et al.*, 2004; Koekemoer *et al.*, 2006). Samples from southern Angola, the FUMOZ laboratory colony, southern Mozambique and South Africa analysed for Restriction Fragment Length Polymorphisms (RFLP) clustered together as the MW-type, suggesting genetic similarity (Koekemoer *et al.*, 2006). Cross-mating experiments between the FUMOZ-R and FANG strains presented in Chapter 3 of this study and also by Ntomwa (2004) showed that hybrid F1 progeny of both sexes are fertile and

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viable. Wondji *et al.* (2007b) identified nine Single Nucleotide Polymorphisms (SNPs) in the genomic sequence of FUMOZ-R, FANG and field samples of *An. funestus* from Malawi (accession no. DQ534436) (sample size = 21 comprising of seven specimens of each strain). They reported that nucleotide diversity was not statistically different between the two laboratory strains (FUMOZ-R and FANG) and field collected mosquitoes despite an apparent low level of heterozygosity observed in the two laboratory strains compared with the field sample. The low level of polymorphism reported by Wondji *et al*. (2007b) supports the results obtained in this study where a high sequence identity was found between the FANG and FUMOZ-R strains.

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CHAPTER 5

DETERMINATION OF PYRETHROID/DDT RESISTANCE MECHANISMS IN A FIELD POPULATION OF *AN. FUNESTUS* FROM GHANA

5.1 INTRODUCTION

Pyrethroid and DDT resistance has been documented in many *Anopheles* species. Target site insensitivity as a result of a single point mutation in the domain II segment 6 of the sodium channel gene has been implicated as the predominant mechanism of resistance. Mutations in this region have been identified in various *Anopheles* species as detailed in Chapter 1. To date, there have been no reports of the presence of any mutations associated with pyrethroid/DDT resistance in *An. funestus*. Bioassay tests for DDT and pyrethroid resistance in a Ghanaian field population of *An. funestus* suggested the presence of the *kdr*-type mutation (unpublished data). In this chapter, the aim was to sequence and screen the sodium channel gene of Ghanaian *An. funestus* for any *kdr*-type mutation(s) that may be associated with resistance to DDT and pyrethroids (which share a similar mode of action (Bloomquist, 1996). It also aimed to identify other mechanisms of resistance in this population using bioassay and biochemical data.

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5.2 MATERIALS AND METHODS

5.2.1 Field population of *An. funestus*

Samples were collected by Prof. M. Coetzee from Obuasi, Ghana in February 2005. Obuasi is a gold-mining community in the Ashanti District, south-central Ghana (06°15′ N, 01°36′ E). Adult mosquitoes were collected resting indoors. Wild caught mosquitoes were initially identified morphologically using the standard taxonomic keys of Gillies & Coetzee (1987).

Wild caught female mosquitoes were transported to the Vector Control Reference Unit (VCRU) insectary and females were induced to lay eggs. Larvae from each family were reared through to adults. Details on rearing and maintenance of mosquitoes are described in Chapter 2. Male and female progeny of the F1 generation from each family were separated on emergence. The progeny by family were divided into two cohorts; one cohort was subjected to standard WHO insecticide bioassays while the other cohort was stored at -70°C for subsequent biochemical analysis of those enzyme families usually associated with insecticide resistance.

The species identity of all *An. funestus* group mosquitoes used in the study was confirmed by PCR using the procedure of Koekemoer *et al.* (2002) (see Chapter 4).

5.2.2 Insecticide bioassays

The bioassay results were kindly provided by Dr LL Koekemoer. The level of resistance in each family was determined by exposing samples of 2 – 3 day old F1 mosquitoes to 4% DDT and/or 0.75% permethrin for 1 h according to WHO (1998) protocols (see Chapter 3). Progeny of mixed families were also exposed to 0.05% deltamrethrin for 1 h. Knockdown was recorded after 1 h and mortality was recorded at 24 h post exposure.

Samples of F1 adults from each family were first exposed to DDT and then samples of remaining larvae from those families that showed significant resistance were pooled and reared to adults. The adults from this pool were labelled as ROF and were exposed to permethrin in order to gain an indication of cross-resistance. The same was done for the susceptible families which were labelled as SOF. Survivors (DDT survivors, permethrin survivors as well as DDT and permethrin survivors) and DDT/permethrin susceptible mosquitoes from the bioassays were stored dry on silica gel and subsequently used for sodium channel gene analysis.

5.2.3 Biochemical analysis

Biochemical assays were carried out in order to determine levels of monooxygenases, non-specific (α and β) esterase, glutathione S-transferase (GST) activity and to detect the presence of altered acetylcholinesterase in 412 F1 adult mosquitoes. Biochemical

assays were performed on 1 - 3 day old frozen female mosquitoes using the method described by Penilla *et al.* (1998) (see Appendix D for details). Analysis included samples from 39 families and sample sizes ranged from 3 to 19 individuals per family. Each microtitre plate contained 10 susceptible (FANG) females (see Chapter 2 for details on FANG strain) which were used for comparative purposes. Blanks were included in each plate as a negative control.

Data from each microtitre plate were standardized by comparing the protein levels in the FANG sample and adjusting the data of the test samples accordingly. Statistical software used in the analysis of data was Statistix 7®. Statistical analyses were carried out using two sample t-tests taking $P<0.05$ by means of 95% confidence. The mean enzyme level of each family (adjusted for total protein content where necessary) was compared to the corresponding FANG mean. The percentage acetylcholinesterase inhibition by propoxur was calculated for each sample by subtracting the percentage uninhibited acetylcholinesterase from 100.

5.2.4 Analysis of domain II of the sodium channel gene of a Ghanaian field population of *An. funestus*

The S5 – S6 segment of the domain II region of the sodium channel gene of a Ghanaian field population of *An. funestus* was PCR amplified (using genomic DNA as template), cloned and sequenced from single mosquitoes as described in Chapter 4. PCR reactions were carried out using two primer sets (N1 and N4; and N3 and N4).

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A total of 12 DDT survivors, 11 DDT and permethrin survivors and 3 DDT/permethrin susceptible mosquitoes were analysed.

The sequences of the sodium channel gene in the Ghanaian population of *An. funestus* were compared to pyrethroid resistant (FUMOZ-R) and susceptible (FANG) laboratory strains (described in Chapter 4) and also to that of *An. gambiae* (Weill *et al.*, 2000).

5.3 RESULTS

5.3.1 Bioassay analysis

5.3.1.1 Bioassays with 4% DDT

Forty-six families were exposed to 4% DDT for 1 h (Table 5.1). The overall percentage mortality across all families was 91.7% (738/805). Ten families (21.7%) (OF 1, OF 8, OF 20, OF 27, OF 28, OF 36, OF 37, OF 39, OF 42 and OF 43) were scored as DDT resistant because they had mortality levels below 80% (WHO, 1998) (Table 5.1). However, there were indications of low levels of DDT resistance (mortality ranging between 80% and 99%) in 8/46 families (17.4%) tested. Hundred percent mortality to 4% DDT was recorded in 30 families (65.2%).

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5.3.1.2 Bioassays with pyrethroids

Unselected mixed families exposed for 1 h to 0.05% deltamethrin produced no survivors (0/43) (Table 5.2). Table 5.3 details the permethrin bioassay results of individual families as well as pooled families of F1 populations of *An. funestus* that previously survived exposure to DDT. Family OF16 and ROF showed high levels of permethrin resistance. The mortality level across all families to permethrin was 89.1% (106/119).

Table 5.1: WHO susceptibility tests using 4% DDT exposures for 1 h against F1 progeny of families generated from a field population of *An. funestus* from Ghana. Sixty minute knock down (KD) and 24 h post exposure mortalities are given.

Family	Males	Females	60 min KD	24 h dead	24 h alive	Total	% mortality
OF 1*	9	7	13	12	4	16	75.0
OF 2	11	12	23	23	0	23	100.0
OF 3	7	4	11	11	0	11	100.0
OF 4	5	5	9	10	0	10	100.0
OF 5	8	2	9	10	0	10	100.0
OF 6	5	8	12	13	0	13	100.0
OF 7	14	7	18	21	0	21	100.0
OF 8*	9	14	16	17	6	23	73.9
OF 9	12	11	21	23	0	23	100.0
OF 10	12	6	18	18	0	18	100.0
OF 11	12	12	23	24	0	24	100.0
OF 12	11	10	20	21	0	21	100.0
OF 13	4	2	6	6	0	6	100.0
OF 14	14	4	16	16	2	18	88.9
OF 15	9	12	19	21	0	21	100.0
OF 16	10	4	11	13	1	14	92.9

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Family	Males	Females	60 min KD	24 h dead	24 h alive	Total	% mortality
OF 17	14	9	21	22	1	23	95.7
OF 18	10	8	17	18	0	18	100.0
OF 19	9	8	15	17	0	17	100.0
OF 20*	4	9	12	9	4	13	69.2
OF 21	8	8	16	16	0	16	100.0
OF 22	11	8	17	19	0	19	100.0
OF 23	5	6	10	11	0	11	100.0
OF 24	13	11	23	23	1	24	95.8
OF 25	18	18	33	34	2	36	94.4
OF 26	18	11	28	29	0	29	100.0
OF 27*	15	14	21	20	9	29	69.0
OF 28*	7	7	12	11	2	14	78.6
OF 29	7	3	9	9	1	10	90.0
OF 30	4	9	11	13	O	13	100.0
OF 31	10	14	22	24	0	24	100.0
OF 32	9	11	17	19	1	20	95.0
OF 33	15	11	22	26	0	26	100.0
OF 34	5	9	14	14	0	14	100.0
OF 35	10	6	17	16	0	16	100.0
OF 36*	15	14	19	22	7	29	75.9
OF 37*	9	16	9	18	7	25	72.0
OF 38	1	1	2	2	0	2	100.0
OF 39*	12	11	12	13	10	23	56.5
OF 40	19	2	21	21	0	21	100.0
OF 41	2	3	5	5	0	5	100.0
OF 42*	12	7	13	14	5	19	73.7
OF 43*	3	4	5	4	3	7	57.1
OF 44	12	6	12	18	0	18	100.0
OF 45	6	3	8	9	0	9	100.0
OF 46	2	1	2	3	0	3	100.0
Total	437	368	691	738	66	805	91.7

(*) indicates families showing <80% mortality 24 h post exposure.

Table 5.2: WHO susceptibility tests using 0.05% deltamethrin for 1 h against F1 progeny from mixed families generated from a field population of *An. funestus* from Ghana. Sixty minute knock down (KD) and 24 h post exposure mortalities are given.

Family	60 min KD	24 h dead	24 h alive	Total	% mortality
mixed	18	19	0	19	100
mixed	17	24	0	24	100
Total	35	43	0	43	100

Table 5.3: WHO susceptibility tests using 0.75% permethrin for 1 h against F1 progeny of families generated from a field population of *An. funestus* from Ghana. Sixty minute knock down (KD) and 24 h post exposure mortalities are given.

Family	Males	Females	60 min KD	24 h dead	24 h alive	Total	% mortality
OF 1	4	6	10	9	1	10	90.0
OF 20	2	8	10	9	1	10	90.0
OF 25	13	17	30	29	1	30	96.7
OF16*	3	1	4	3	1	4	75.0
OF36	8	8	16	15	1	16	93.8
ROF	0	24	24	18	6	24	75.0
SOF	0	25	25	23	2	25	92.0
Total	30	89	119	106	13	119	89.1

ROF indicates pooled individuals of families that survived previous exposure to DDT. SOF indicates pooled individuals of families previously shown to be susceptible to DDT. (*) indicates families with <80% mortality 24 h post exposure.

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5.3.2 Biochemical analysis

5.3.2.1 α esterase activity

Figure 5.1 shows the level of α esterase activity per family with the FANG base line set at zero. Biochemical analysis of sub samples of F1 progeny revealed that there was an increased α esterase level in 28 out of the 39 families (71.8%) tested relative to the levels recorded for the susceptible FANG females. The α esterase level was significantly higher in 17 families (43.6%) (Table 5.4).

5.3.2.2 β esterase activity

Figure 5.2 shows the level of β esterase activity per family with the FANG base line set at zero. Biochemical analysis of sub samples of F1 progeny revealed that there was an increased β esterase level in 25 out of the 37 families (67.6%) tested relative to the levels recorded for the susceptible FANG females. β esterase levels were significantly higher in 20 families (54.1%) (Table 5.4).

Figure 5.1: Percentage increase or decrease in α esterase levels of listed *An. funestus* families from Ghana relative to the susceptible reference colony FANG. (*) indicates families resistant to either DDT or pyrethroids by bioassay.

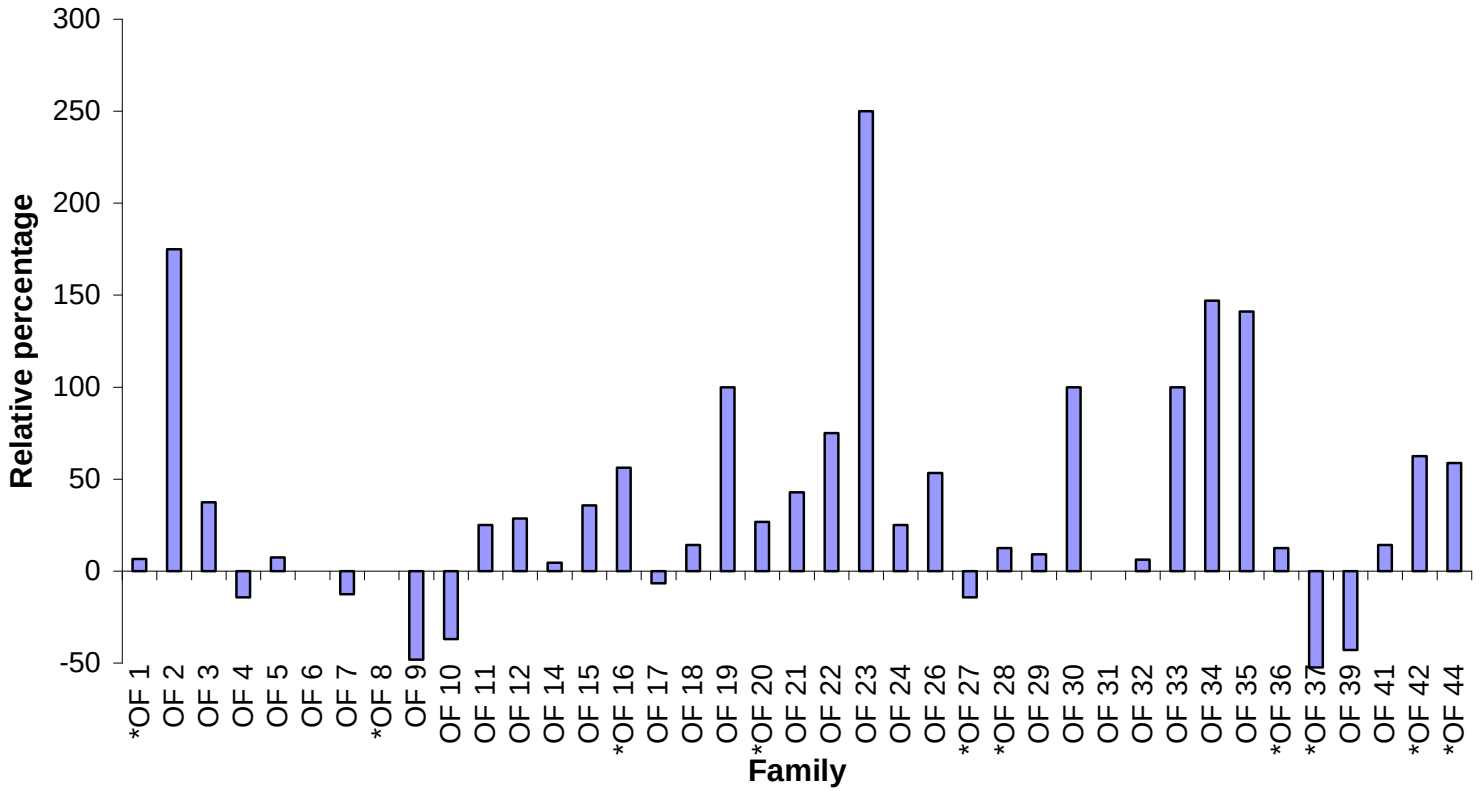
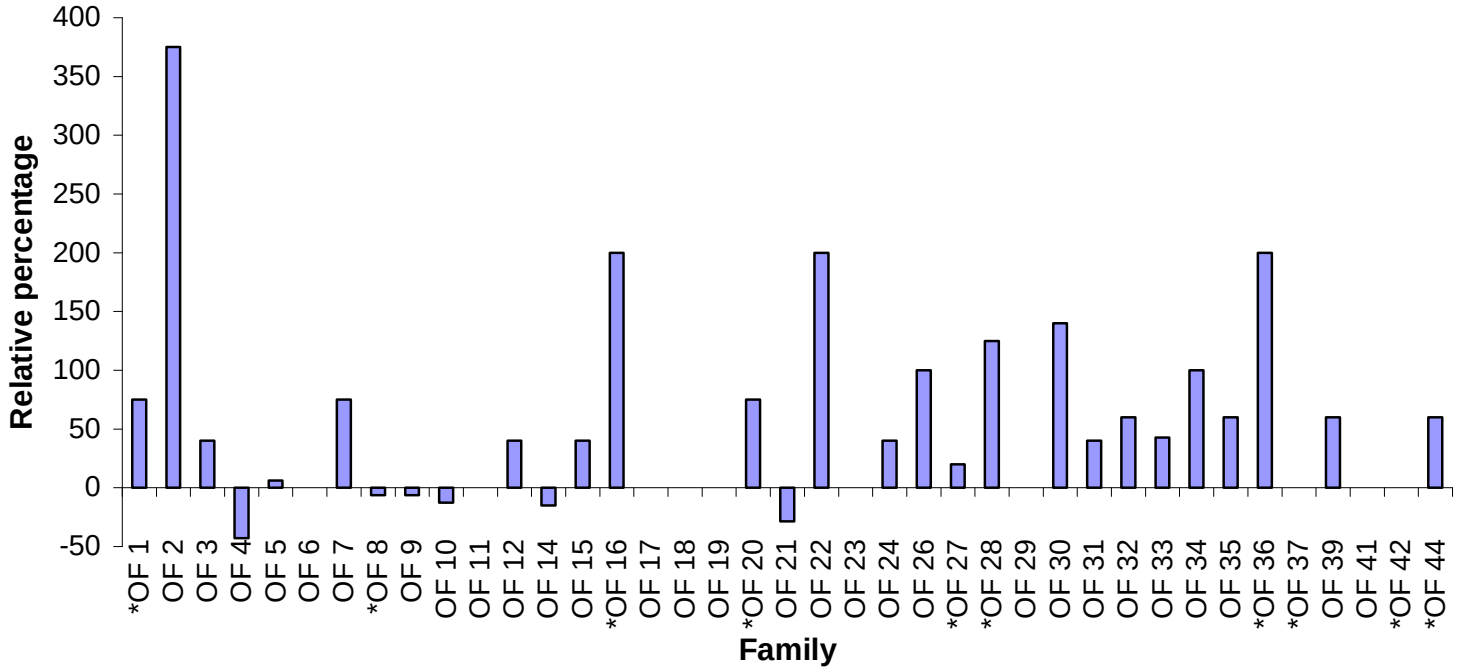


Figure 5.2: Percentage increase or decrease in β esterase levels of listed *An. funestus* families from Ghana relative to the susceptible reference colony FANG. (*) indicates families resistant to either DDT or pyrethroid by bioassay.



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5.3.2.3 Monooxygenase titration

Figure 5.3 shows the relative titre of monooxygenases per family with the FANG baseline set at zero. There was an increased monooxygenase level in 18 out of the 39 families (46.2%) tested relative to the level recorded for the susceptible FANG females. However, there was a significant increase in only 5 of these families (12.8%) (Table 5.4).

5.3.2.4 Glutathione S-Transferase (GST) activity

Figure 5.4 shows the level of GST activity per family with the FANG base line set at zero. Biochemical analysis of sub samples of F1 progeny revealed that there was an increased GST level in 16 out of the 39 families (41%) examined relative to the levels recorded for the susceptible FANG females. However, this was only significantly higher in 6 of these families (15.4%) (Table 5.4).

Figure 5.3: Percentage increase or decrease in monooxygenase levels of *An. funestus* families from Ghana relative to the susceptible reference colony FANG. (*) indicates families resistant to DDT or pyrethroids by bioassay.

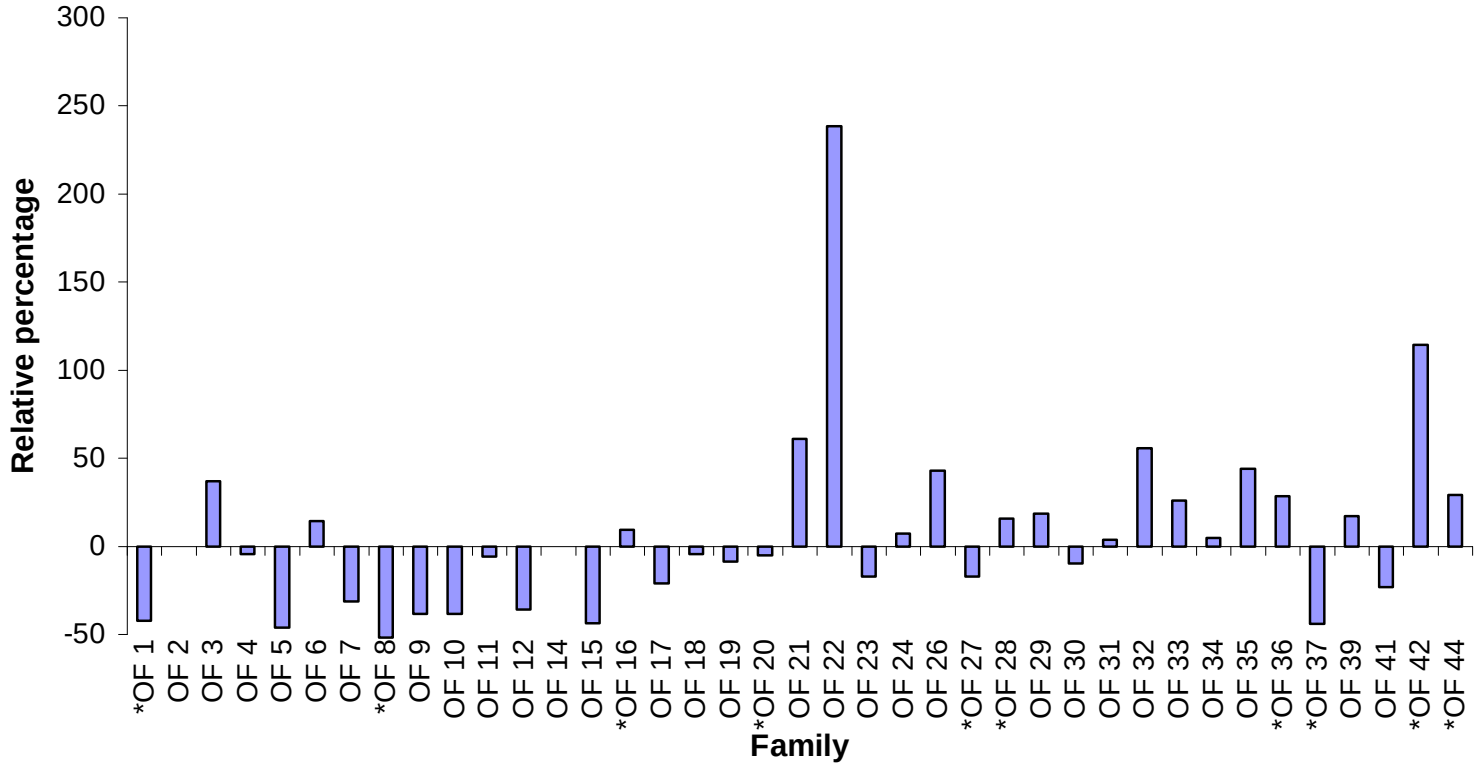

Figure 5.4: Percentage increase or decrease in GST levels of listed *An. funestus* families from Ghana relative to the susceptible reference colony FANG. (*) indicates families resistant to either DDT or pyrethroids by bioassay.

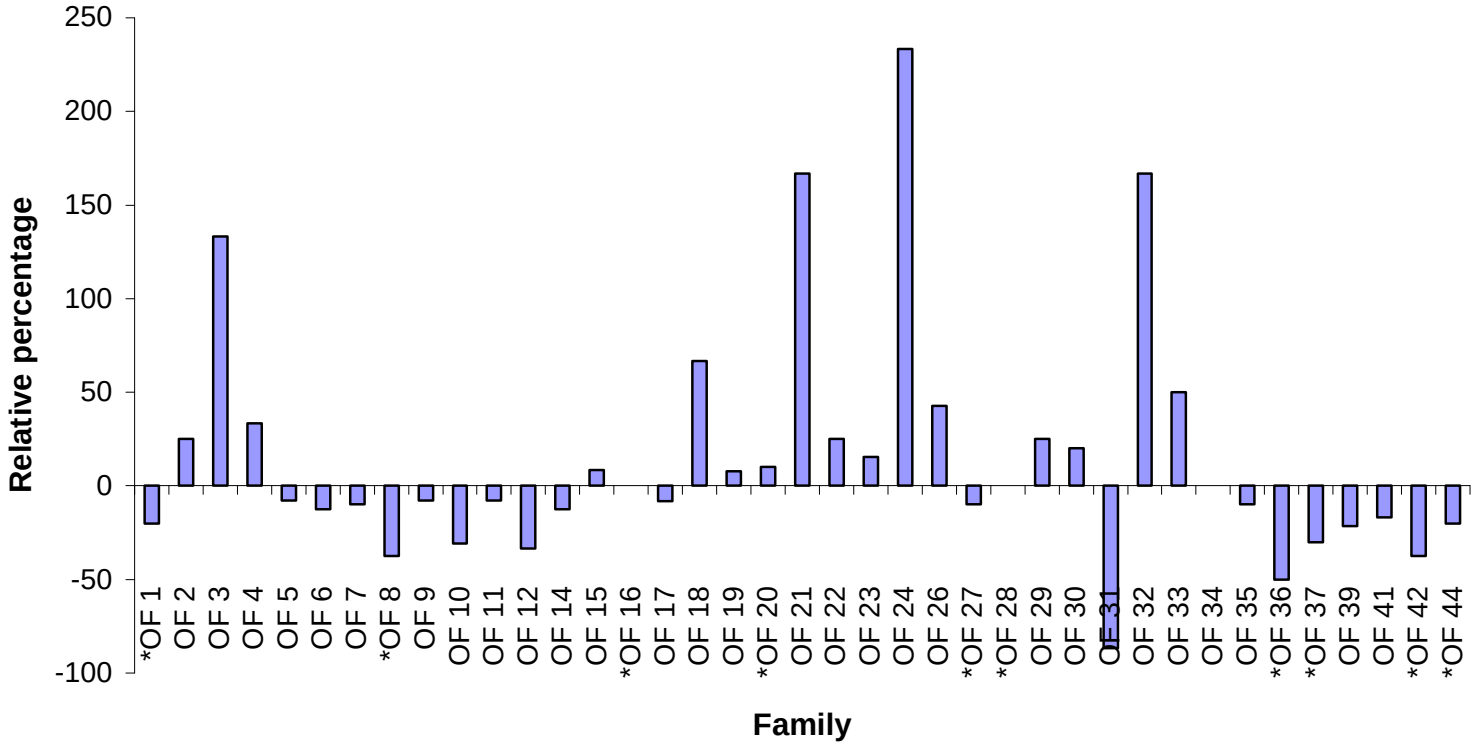

Table 5.4: Comparison of optical density (OD) values between the F1 progeny of *An. funestus* families generated from wild-caught females from Ghana and their corresponding baseline controls (FANG). Mean OD values (± s.e.) are given per enzyme system.

Family	n	Monooxyge-nases	GST	α- esterase	β- esterase
OF 1[a]	10	0.22 ± 0.02	0.08 ± 0.0009	0.16 ± 0.006	0.07 ± 0.003*
OF 2	6	0.07 ± 0.014	0.01 ± 0.0008*	0.22 ± 0.03*	0.19 ± 0.008*
OF 3	10	0.37 ± 0.05	0.007 ± 0.002	0.22 ± 0.01*	0.07 ± 0.004
OF 4	4	0.22 ± 0.03	0.004 ± 0.001	0.12 ± 0.006	0.04 ± 0.005
OF 5	14	0.14 ± 0.03	0.012 ± 0.0005	0.29 ± 0.01	0.17 ± 0.03
OF 6	12	0.08 ± 0.03	0.007 ± 0.0004	0.08 ± 0.007	0.04 ± 0.006
OF 7	15	0.22 ± 0.03	0.009 ± 0.0006	0.14 ± 0.014	0.07 ± 0.0097*
OF 8[a]	10	0.13 ± 0.02	0.005 ± 0.001	0.22 ± 0.009	0.044 ± 0.003
OF 9	10	0.16 ± 0.02	0.012 ± 0.0006	0.14 ± 0.02	0.15 ± 0.01
OF 10	14	0.16 ± 0.02	0.009 ± 0.0005	0.17 ± 0.01	0.14 ± 0.009
OF 11	15	0.24 ± 0.02	0.005 ± 0.0006	0.16 ± 0.008	0.07 ± 0.004*
OF 12	12	0.25 ± 0.02	0.004 ± 0.0009	0.18 ± 0.01*	0.07 ± 0.01
OF 14	14	0.27 ± 0.04	0.007 ± 0.0008	0.23 ± 0.01	0.04 ± 0.003
OF 15	13	0.22 ± 0.02	0.0065 ± 0.0008	0.19 ± 0.14*	0.07 ± 0.006*
OF 16[a]	6	0.35 ± 0.04	0.01 ± 0.0017	0.25 ± 0.02*	0.12 ± 0.015*
OF 17	12	0.3 ± 0.04	0.011 ± 0.001	0.14 ± 0.009	0.04 ± 0.004
OF 18	11	0.22 ± 0.03	0.005 ± 0.0008	0.16 ± 0.02	0.07 ± 0.01
OF 19	15	0.32 ± 0.04	0.014 ± 0.0005	0.08 ± 0.02	-
OF 20	12	0.36 ± 0.02	0.011 ± 0.0012	0.19 ± 0.008*	0.07 ± 0.004*
OF 21	3	0.37 ± 0.04*	0.008 ± 0.002*	0.2 ± 0.01*	0.05 ± 0.005*
OF 22	6	0.237 ± 0.05*	0.01 ± 0.002	0.14 ± 0.03*	0.12 ± 0.03*
OF 23	15	0.29 ± 0.01	0.015 ± 0.0003*	0.14 ± 0.03*	-
OF 24	14	0.29 ± 0.03	0.01 ± 0.002*	0.2 ± 0.01*	0.07 ± 0.002*
OF 26	14	0.4 ± 0.05	0.01 ± 0.001*	0.23 ± 0.01*	0.08 ± 0.006*
OF 27[a]	19	0.34 ± 0.03	0.009 ± 0.0008	0.18 ± 0.01	0.06 ± 0.04
OF 28[a]	14	0.37 ± 0.044	0.01 ± 0.0005	0.18 ± 0.007	0.09 ± 0.009*
OF 29	13	0.32 ± 0.05	0.01 ± 0.001	0.24 ± 0.01	0.05 ± 0.0045
OF 30	14	0.37 ± 0.02	0.012 ± 0.0007	0.34 ± 0.01*	0.24 ± 0.02*

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Family	n	Monooxyge-nases	GST	α- esterase	β- esterase
OF 31	3	0.28 ± 0.01	0.004 ± 0.002	0.16 ± 0.01	0.07 ± 0.002*
OF 32	12	0.42 ± 0.04*	0.08 ± 0.002*	0.17 ± 0.01	0.08 ± 0.007*
OF 33	10	0.29 ± 0.02	0.0045 ± 0.002	0.28 ± 0.03*	0.1 ± 0.02
OF 34	3	0.43 ± 0.01	0.01 ± 0.002	0.42 ± 0.03*	0.2 ± 0.02*
OF 35	7	0.59 ± 0.05*	0.009 ± 0.0004	0.41 ± 0.03*	0.16 ± 0.02*
OF 36[a]	10	0.09 ± 0.018	0.004 ± 0.0006	0.09 ± 0.016	0.12 ± 0.02*
OF 37[a]	4	0.23 ± 0.03	0.007 ± 0.0005	0.1 ± 0.01	0.05 ± 0.009
OF 39[a]	12	0.48 ± 0.04	0.011 ± 0.001	0.12 ± 0.02	0.08 ± 0.01*
OF 41	4	0.3 ± 0.07	0.005 ± 0.001	0.16 ± 0.02	0.05 ± 0.005
OF 42[a]	8	0.15 ± 0.04*	0.005 ± 0.001	0.13 ± 0.02*	0.04 ± 0.01
OF 44	12	0.53 ± 0.04	0.01 ± 0.001	0.27 ± 0.02*	0.16 ± 0.01*

(*) indicates family value significantly higher than susceptible strain ($p<0.05$). ([a]) indicates family that showed resistance to either DDT or pyrethroids by bioassay.

5.3.2.5 Acetylcholinesterase inhibition

Table 5.5 shows the results of propoxur inhibition of acetylcholinesterase for the F1 progeny of families of *An. funestus* from Ghana. Thirty out of the 39 families (76.9%) examined showed a significantly lower percentage of propoxur inhibition of acetylcholinesterase compared to the susceptible strain (FANG).

Table 5.5: Mean percentage acetylcholinesterase inhibition by propoxur of the F1 progeny of families of *An. funestus* from Ghana.

Family	Sample size (n)	Mean % inhibition ± s.e
OF 1	10	46.2 ± 3*
OF 2	3	5.6 ± 5.6*
OF 3	9	27.4 ± 2.7*
OF 4	4	16.9 ± 4.8*
OF 5	13	45.2 ± 3.9*
OF 6	11	60.8 ± 5.7
OF 7	12	48.1 ± 8.1
OF 8	10	42 ± 2.5*
OF 9	10	25 ± 2.7*
OF 10	14	41 ± 3.3*
OF 11	11	36 ± 4.6*
OF 12	12	29 ± 4.1*
OF 14	14	54.1 ± 3.5*
OF 15	11	27.5 ± 1.7*
OF 16	6	57.6 ± 3.7
OF 17	11	54.5 ± 3.9*
OF 18	11	31.2 ± 3.2*
OF 19	15	32.3 ± 6.9*
OF 20	6	14.9 ± 6.2*
OF 21	11	26.7 ± 5.8*
OF 22	6	61.1 ± 6.1
OF 23	13	37.8 ± 4.6*
OF 24	14	24.2 ± 6.5*
OF 26	12	46.4 ± 4.2*
OF 27	19	65 ± 3.3
OF 28	7	51.4 ± 10.3
OF 29	12	31.7 ± 3.3*
OF 30	14	42.6 ± 3.9*
OF 31	3	25 ± 3.6*
OF 32	11	40.3 ± 4.2*
OF 33	10	30.5 ± 1.6*
OF 34	3	34.1 ± 1.6*
OF 35	7	32.6 ± 4*
OF 36	9	42 ± 4.9*
OF 37	4	63.9 ± 2.7

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Family	Sample size (n)	Mean % inhibition ± s.e
OF 39	12	57.3 ± 3.2
OF 41	4	36.5 ± 6.7*
OF 42	8	57.4 ± 6.1
OF 44	9	27.8 ± 6.2*
FANG	10	66.1 ± 3

(*) indicate significantly lower inhibition than susceptible strain ($p<0.05$).

5.3.3 Analysis of domain II of the sodium channel gene of a Ghanaian field population of *An. funestus*.

PCR amplification of the sodium channel gene of samples from the Ghana field populations of *An. funestus* using N3 and N4 primers yielded a 238 bp product while N1 and N4 primers yielded a product size of 1423 bp. The entire sequence has been deposited in GenBank (accession no. EF687845). Alignment of the four sets of sequences obtained from the DDT survivors, permethrin survivors, DDT and permethrin survivors and the DDT/permethrin susceptible mosquitoes showed 100% homology. Figure 5.5 shows an alignment of the genomic sequences of DDT/pyrethroid resistant field populations of *An. funestus* from Ghana against the pyrethroid resistant (FUMOZ-R) and pyrethroid susceptible (FANG) laboratory strains and *An. gambiae* (obtained from Weill *et al.*, 2000, no accession number given).

Alignment of the nucleotide sequences obtained from the Ghanaian samples revealed a sequence identity of 99.4%, 99.5% and 68.5% to FUMOZ-R, FANG and *An.*

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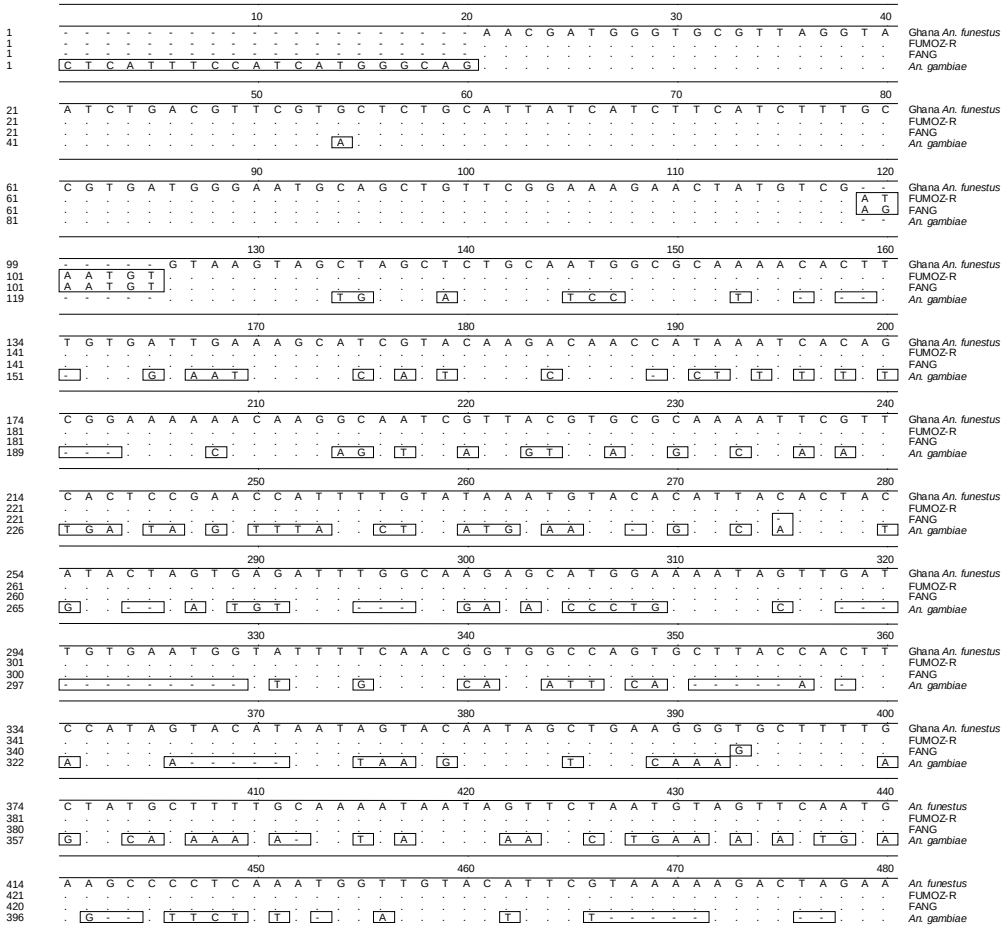
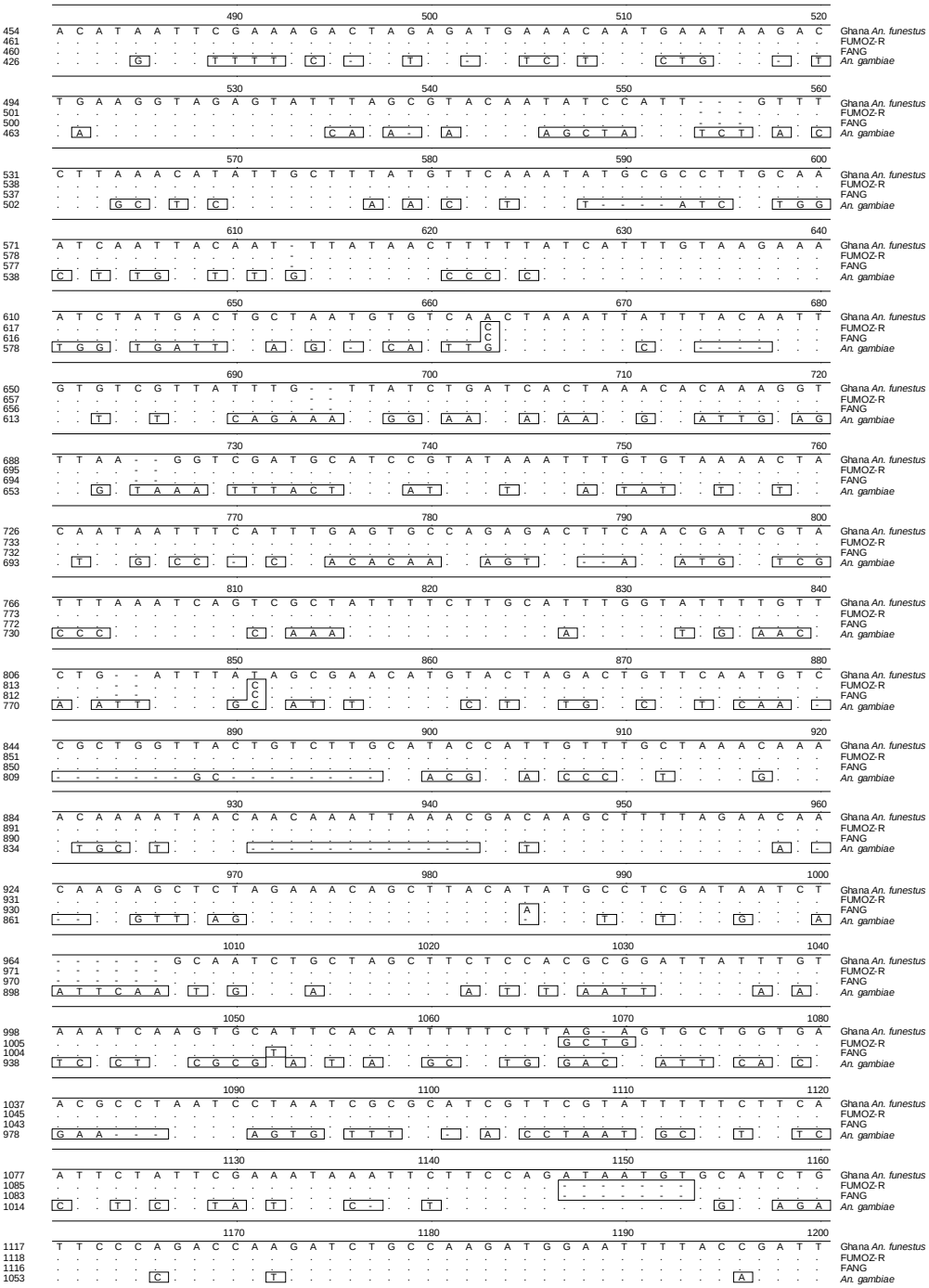
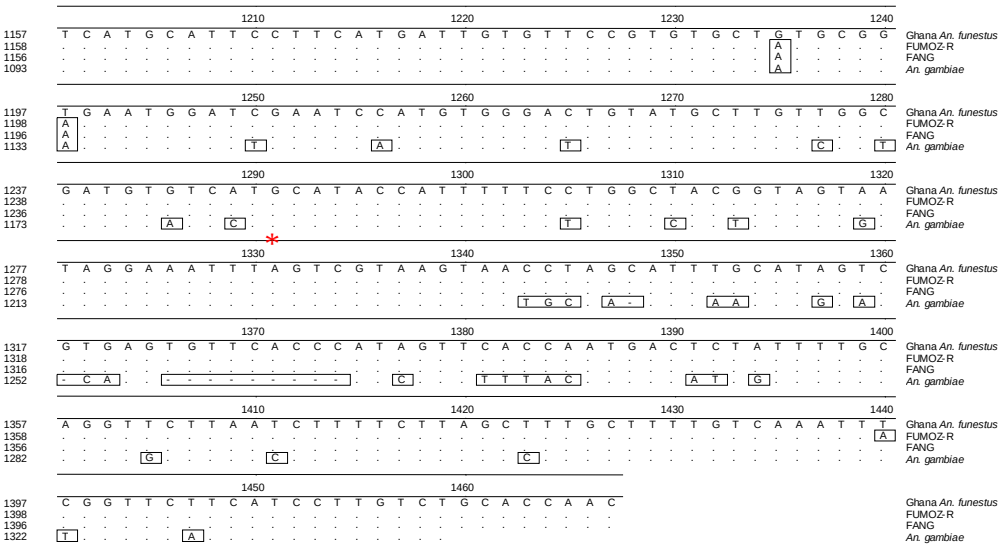
gambiae (obtained from Weill *et al.*, 2000) respectively. Interestingly, there was a deletion of 5'ATAATGT3' from position 99 - 105 (nucleotide position numbering of FUMOZ-R) in the Ghanaian *An. funestus* samples when compared to the same position in FUMOZ-R and FANG. This same segment was inserted at position 1103 - 1109 (nucleotide position numbering of FUMOZ-R) in the Ghanaian *An. funestus* samples, from where it was absent in FUMOZ-R. The *kdr* mutation (mutation of an adenine (A) to thymine (T) at the *kdr* mutation position (indicated in Figure 5.5)) which had been reported in other insects including *An. gambiae* (Martinez-Torres *et al.*, 1998), *An. arabiensis* (Verhaeghen *et al.*, 2006; Matambo *et al.*, 2007) and *An. sacharovi* (Lüleyap *et al.*, 2002) was absent in the Ghanaian *An. funestus* population.

Figure 5.5: Alignment of the genomic sequences of the IIS5 - IIS6 region of the voltage-sensitive sodium channel of DDT/pyrethroid resistant *An. funestus* field samples from Ghana against the pyrethroid resistant (FUMOZ-R) and pyrethroid susceptible (FANG) laboratory strains of *An. funestus* and *An. gambiae* (obtained from Weill *et al.*, 2000). Identical positions are indicated by a dot (.) and differences are indicated by a box. The supposedly *kdr* mutation is indicated below *.



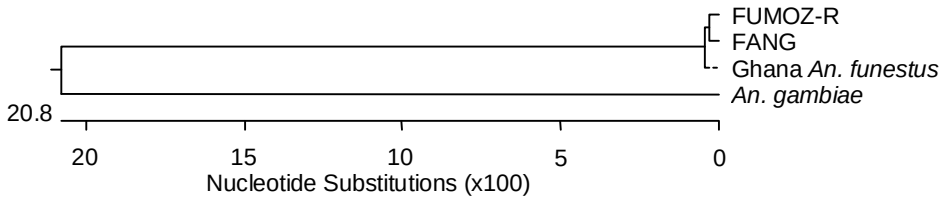




5.3.3.1 Phylogenetic analysis

Figure 5.6 shows the phylogenetic relationship between the Ghanaian *An. funestus* samples and the FUMOZ-R and FANG laboratory colonies and *An. gambiae* (obtained from Weill *et al.*, 2000). The phylogenetic tree shows that the Ghanaian samples are more closely related to FUMOZ-R and FANG than to *An. gambiae*.

Figure 5.6: Phylogenetic relationship between the genomic sequence of the IIS5 - IIS6 region of the voltage-sensitive sodium channel in DDT/pyrethroid resistant *An. funestus* field populations from Ghana, the pyrethroid resistant (FUMOZ-R) and pyrethroid susceptible (FANG) laboratory strains of *An. funestus* and *An. gambiae* (obtained from Weill *et al.*, 2000).



5.4 DISCUSSION

5.4.1 Bioassays

Using the WHO (1998) bioassay protocol, a sample is considered resistant if more than 20% of the mosquitoes exposed survive the standard diagnostic dose for any given insecticide. The bioassay results using WHO discriminating dosages showed that all families were completely susceptible to deltamethrin. However, some families were resistant to DDT and/or permethrin. When the F1 family progeny that had previously survived DDT were pooled and exposed to permethrin, 25% survival was recorded indicating that there may be cross-resistance. Cross-resistance between DDT and pyrethroids is quite common and has been reported in *An. gambiae* (Elissa *et al.*,

1993), *An. minimus* (Chareonviriyaphap *et al*., 2002), *Ae. aegypti* (Brealey *et al.*, 1984) and *An. stephensi* (Omer *et al.*, 1980).

High levels of DDT and pyrethroid resistance were recorded in some families of Ghanaian *An. funestus*. Low levels of resistance to DDT and pyrethroids were observed across most families. It should be noted that these bioassays cannot be used to measure resistance gene frequencies. They are best used as good indicators of the presence of significant resistance in a mosquito population (Penilla *et al*., 1998). Brooke *et al*. (1999) emphasized the importance of using both molecular and biochemical techniques to verify bioassay results for the detection of resistance in wild populations since the bioassay test alone does not provide information on the underlying mechanisms of resistance.

5.4.2 Biochemical assays

Most of the F1 families studied had elevated enzyme levels. Out of the 39 families analyzed, 92.3% (36/39) gave indications of either monooxygenase, GST, α esterase or β esterase elevation or presence of an altered acetylcholinesterase. Most families (76.9%) gave indications of the presence of an altered acetylcholinesterase. Elevated β and α esterases level was recorded in 54.1% and 43.6% of the families respectively while GST (15.4%) and monooxygenase (12.8%) elevation was indicated in a few families. High levels of all four enzymes as well as the presence of an altered acetylcholinesterase were recorded in only one family (OF 21).

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Monooxygenases and esterases have been implicated in pyrethroid resistance, while elevated GST levels have been implicated in DDT resistance (Prapanthadara *et al.*, 1995; Brogdon *et al.*, 1999; Brooke *et al.*, 2001; Enayati *et al.*, 2003). Interestingly most of the families that had indicated complete susceptibility to DDT based on bioassays also had elevated enzyme levels. Only six out of the nine families (OF 1, OF 20, Of 28, OF 36, OF 42 and OF 39) that indicated high DDT resistance by bioassay had elevated enzyme levels. Only one family (OF 16) that indicated permethrin resistance based on bioassay data also showed elevated enzyme levels. Of the bioassay resistant families, β esterases were elevated in OF 1 and OF 28; α and β esterases were elevated in OF 20 and OF 16 while monooxygenases and α esterases showed elevated levels in OF 42. No elevated GST levels were recorded in any of these bioassay resistant families. Generally, there was no significant correlation between enzyme level and insecticide susceptibility data.

The few families that recorded high GST (6/39) or monooxygenase (5/39) levels seemed to do so in conjunction with comparatively high levels of either α esterases and/or β esterases. This suggests that insecticide detoxification based on multiple enzyme elevation is the cause of the resistance phenotypes recorded.

Carbamate and organophosphate insecticides target acetylcholinesterase (AChE) (Hemingway, 1989). The low percentages of propoxur inhibition obtained for most families suggests that an altered AChE showing a lower affinity for carbamates is present in the Ghanaian *An. funestus* field population. Progeny from these families

were not exposed to organophosphates and carbamates because the original aim of the study was to determine whether any *kdr*-type mutation was present in the Ghanaian *An. funestus* population. The samples obtained from each of the families were also too small to allow for testing of more than one class of insecticide. However, wild samples of *An. funestus* from Obuasi show 71.4% mortality when exposed to the carbamate bendiocarb (0.1%) for one hour (Coetzee *et al.*, 2006). The biochemical results presented here suggest that this Ghanaian *An. funestus* population is resistant to carbamates.

Elevated esterase levels recorded in most of the Ghanaian *An. funestus* families and the high prevalence of the AChE gene in the population may have arisen as a result of use of insecticides in the localities close to the mosquito collection site. AChE genes give high levels of resistance to carbamates even when individuals are heterozygous for the gene (Hemingway & Smith, 1986). When AChE genes are present in combination with elevated esterase genes they produce a higher level of resistance to some organophosphates than either metabolism on its own (Hemingway & Smith, 1986).

5.4.3 Analysis of the domain II sodium channel gene of a Ghanaian field population of *An. funestus*

The fact that no nucleotide difference was found between the sequences obtained from the DDT survivors, permethrin survivors, DDT/permethrin survivors and the

DDT/permethrin susceptible samples suggests that this region of the sodium channel gene does not play a part in DDT/pyrethroid resistance. The percentage variability within the genomic sequence of the Ghanaian samples compared with FUMOZ-R, FANG and *An. gambiae* (obtained from Weill *et al.*, 2000) was 0.6%, 0.5% and 41.5% respectively. The high similarity observed between the Ghanaian samples and southern African *An. funestus* (FUMOZ-R and FANG) suggests that the function of this gene, and therefore the sequence, is highly conserved in this species. The absence of the known *kdr*-type mutations in the IIS5 - IIS6 of the sodium channel gene does not preclude the possibility of other mutations elsewhere in the sodium channel gene.

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CHAPTER 6

CONCLUSIONS

Most attempts to manage resistance aim to exploit the reduction in fitness of resistant genotypes relative to susceptible genotypes by preserving susceptible homozygotes and eliminating heterozygotes and resistance homozygotes (Leeper, *et al.*, 1986). Pyrethroid resistance in *An. funestus* originating from southern Mozambique and KwaZulu-Natal, South Africa, has been shown to be metabolic, essentially based on the overproduction of monooxygenases (Brooke *et al.*, 2001). The results of the present study suggest that such overproduction does not compromise the overall fitness of individuals carrying the resistance phenotype. The present data are consistent with the resistance being under the control of a single, major, incompletely dominant allele showing a comparatively high degree of fitness and stability in the presence or absence of insecticide selection and can thus be expected to spread readily within and between populations where pyrethroids are used. Consequently, the frequency of the resistance genes would probably not decline in the absence of insecticide pressure since pyrethroid resistant mosquitoes would be expected to compete favourably with wild-type susceptibles. It follows that insecticide resistance management strategies assuming relative fitness disadvantage of resistance phenotypes would not succeed for pyrethroid resistant *An. funestus* populations in southern Africa. In the absence of strong natural selection against the resistance genes

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one cannot expect a major benefit from the immigration of susceptible individuals from untreated surrounding areas or untreated refuges within the target area.

The implications of these results for insecticide resistance management in affected regions suggest that control authorities cannot assume that relaxation of pyrethroid selection pressure will translate into a significant decrease in the frequency of resistance alleles in affected *An. funestus* populations. Evidence of cross-resistance to carbamate insecticides (Brooke *et al*., 2001) complicates the situation further, leaving organochlorines (such as DDT) and organophosphate insecticides as the best available options. A holistic malaria control programme should include the rotational use of demonstrably effective insecticides, non-chemical control methods (Georghiou & Taylor, 1986) as well as treatment and prophylactic prevention of malaria infection. This information requires careful consideration in terms of designing an effective resistance management strategy to combat insecticide resistant *An. funestus* populations in southern Africa.

This study also provides information concerning the resistance mechanism/s involved in a DDT/pyrethroid and carbamate resistant population of *An. funestus* from Obuasi, Ghana. Molecular studies showed that *kdr*-type mutations were absent in the IIS5 - IIS6 segment of the sodium channel gene of both southern African and Ghanaian *An. funestus* indicating that pyrethroid resistance is primarily metabolic in both cases. However, pyrethroid/DDT detoxification in the Ghanaian population is most likely based on the elevated activity of a cocktail of detoxifying enzyme systems. In

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addition, the presence of an altered acetylcholinesterase conferring carbamate resistance in the Ghanaian *An. funestus* population indicates that insecticide resistance in this population is clearly different from that of southern African *An. funestus* where pyrethroid and carbamate resistance is primarily based on elevated levels of monooxygenases (Brooke *et al*., 2001).

These findings illustrate the difficulties associated with managing resistance in vector populations because populations belonging to the same vector species may adapt differently to the presence of insecticide in their respective environments. They confirm that there is no simple, universally applicable form of vector control because of the wide variety of life history strategies employed by malaria vectors (Collins & Paskewitz, 1995). Choice of vector control strategy should be based on the biological characteristics of local vectors including their responses to insecticide exposure as well as the effects of resistance genes on biological fitness in the absence of insecticide selection pressure. The results obtained here have implications for the development of resistance management strategies designed to control *An. funestus* populations in Africa.

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APPENDIX A

EXPERIMENTAL SET UP

Figure A.1: Individual mosquitoes isolated in glass vials for egg laying.

Figure A.2: Eggs from single families placed in bowls for hatching.

Figure A.3: Larvae from single families reared in bowls.

Figure A.4: WHO test kits for testing adult anopheline susceptibility to insecticide, insecticide treated papers and an aspirator.

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APPENDIX B

PUBLICATIONS

Okoye, P.N., Brooke, B.D., Hunt, R.H. & Coetzee, M. 2007. Relative developmental and reproductive fitness associated with pyrethroid resistance in the major southern African malaria vector *Anopheles funestus*. *Bulletin of Entomological Research* 97: 599-605.

Okoye, P.N., Brooke, B.D., Koekemoer, L.L., Hunt, R.H. & Coetzee, M. 2008. Inheritance of pyrethroid resistance in the major southern African malaria vector *Anopheles funestus*. *Annals of Tropical Medicine & Parasitology* 102: 275-281.

Okoye, P.N., **Brooke, B.D.**, Koekemoer, L.L., Hunt, R.H. & Coetzee, M. 2008. Characterisation of DDT, pyrethroid and carbamate resistance in *Anopheles funestus* from Obuasi, Ghana. *Transactions of the Royal Society of Tropical Medicine & Hygiene* (in press).

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APPENDIX C

STANDARD PROCEDURES FOR MOLECULAR STUDIES

C.1 DNA Extraction

One day old individual mosquitoes were homogenised with a sterile pestle in 200 µl grinding buffer (1 M NaCl, 5% sucrose, 0.5 M EDTA (pH 7.8), 10% SDS and 1 M Tris-Cl (pH 8.6)). The homogenate was incubated at 70°C for 30 minutes followed by the addition of 28 µl of 8 M potassium acetate. It was incubated on ice for 30 minutes followed by centrifugation for 10 minutes at 13,000 rpm and 400 µl of 100% ethanol was added to the supernatant. The mixture was incubated at -20°C for 60 minutes. The pellet was washed in 200 µl 70% ethanol and centrifuged for 10 minutes at 13,000 rpm. The supernatant was discarded and the DNA pellet air dried for 5 – 10 minutes after which it was re-suspended in 200 µl distilled water. For each set of DNA extractions, a negative control containing all the reagents used in the extraction process except the mosquito DNA was included. Pestles used in homogenizing the mosquito samples and in negative controls were sterilized under ultraviolet light for 30 minutes to one hour before use. Sterilized pestles were stored in a sterile container with a screw – on lid.

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The extracted DNA was purified using phenol/chloroform. The volume of each DNA sample was made up to 400 µl using distilled water. Two hundred µl phenol and 200 µl chloroform was then added and the mixture was mixed and centrifuged at 13,000 rpm. Two hundred µl of chloroform was added to the aqueous layer and the mixture centrifuged for 1 minute at 13,000 rpm. To the aqueous layer, 40 µl 3 M NaAc (pH 4) and 800 µl absolute ethanol was added and the mixture incubated at -20°C for at least one hour after which centrifugation was carried out at 13,000 rpm for 30 minutes. The DNA pellet was washed in 70% ethanol and centrifuged for 5 minutes at 13,000 rpm. The supernatant was discarded and the pellet air dried for 5 – 10 minutes after which it was re-suspended in 50 µl distilled water. The DNA was quantified using a GENE QUANT *pro* (Amersham Biosciences, 89761) or NANODROP (ND 1000) spectrometer at 260/280 nm absorption levels and stored at -20°C until needed.

C.2 *Anopheles funestus* species-specific identification

The PCR reaction mixture consisted of 10 X PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 1.5 mM $MgCl_2$, 3.3 µM of each primer (UV, FUN, VAN, RIV, PAR and LEES), 200 µM of each dNTP, and 0.5 units Takara *Taq* DNA polymerase (Takara Bio Inc. Japan, code R001AM) in a 12.5 µl reaction mixture. The PCR cycle conditions was at an initial denaturation of 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 40 seconds; and a final extension cycle of 72°C for 5 minutes. PCR products were electrophoresed on a 1.5% (w/v) agarose gel in 1 X TAE buffer containing 10 mg/ml

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ethidium bromide (Sigma, cat no. E-1510). Electrophoresis was performed at a constant voltage of 100V in 1 X TAE buffer. Size marker was loaded on the agarose gel.

C.3 Total RNA isolation

Mosquitoes were immobilised briefly at -20^{0}C. Three mosquitoes (one day old) were homogenized in 200 µl of TRI Reagent® (Sigma, cat. no. T9424) and incubated for 5 minutes at room temperature to ensure complete dissociation. To this, 0.2 ml chloroform per ml of TRI Reagent® was added and the sample mixture was shaken vigorously for 15 seconds and incubated for 5 minutes at room temperature. The mixture was then centrifuged (12,000 g, 15 minutes, 4^{o}C), 0.5 ml of room temperature isopropanol per ml of TRI Reagent® was added to the aqueous phase and the mixture was incubated for 10 minutes at room temperature. The mixture was centrifuged (12,000 g, 10 minutes, 4°C) and the pellet was washed in 1 ml of 75% ethanol per ml of TRI Reagent®. This was then centrifuged at 7,500 g for 5 minutes at 4°C and the RNA pellet air dried for 5 - 10 minutes. The pellet was re-suspended in 20 µl of 0.01% (v/v) diethyl pyrocarbonate (DEPC) treated water. Isolated RNA was quantified using a GENE QUANT *pro* (Amersham Biosciences, 89761) or NANODROP (ND 1000) spectrometer at 260/280 nm absorption levels and stored at -70°C until needed.

C.4 cDNA synthesis

cDNA synthesis was carried out using the ImProm-II™ Reverse Transcription System (Promega, USA. cat no. A3800) and positive and negative controls were included. The extracted experimental RNA was thawed on ice. One µg of experimental RNA and 0.5 µg/reaction of Oligo $(dT)_{15}$ were combined in nuclease free water to a final volume of 5 µl per reverse transcription (RT) reaction. The positive control consisted of 0.5 µg/µl of 1.2 kb Kanamycin positive control RNA and 0.5 µg/reaction of Oligo $(dT)_{15}$ made up in nuclease free water to a final volume of 5 µl per RT reaction. The negative control comprised of 0.5 µg/reaction of Oligo $(dT)_{15}$ made up in nuclease free water to a final volume of 5 µl per RT reaction. Each tube was closed tightly and incubated at 70°C for 5 minutes, followed immediately by chilling on ice for 5 minutes. Each tube was centrifuged for 10 seconds to collect the condensate and maintain the original volume. Tubes were kept closed on ice until the reverse transcription reaction mix was added.

The reverse transcription (RT) reaction mix comprised of ImProm-II™ 5X reaction buffer, 1.5 mM $MgCl_2$, 10 mM dNTP mix, recombinant Rnasin Ribonuclease inhibitor and ImProm-II™ reverse transcriptase to a final volume of 15 µl. To this, 5 µl of RNA/primer mixture was added giving a total of 20 µl. Annealing was carried out at 25°C for 5 minutes; followed by extension at 50°C for one hour and the reverse transcriptase was thermally inactivated prior to amplification at 70°C for 15 minutes. The cDNA was quantified using the GENE QUANT *pro* (Amersham Biosciences,

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89761) or the NANODROP (ND 1000) spectrometer and 2 µl of the cDNA was electrophoresed on a 1% (w/v) agarose gel in 1 X TAE buffer containing 10 mg/ml ethidium bromide (Sigma, cat no. E-1510). The remainder of the sample was kept at -20°C until needed.

C.5 Purification of DNA from agarose gel

Hundred µl of PCR product of each sample was electrophoresed on a 1% ethidium bromide stained low melting point agarose gel (Research Organics, USA, cat. no. 1170A). The DNA fragment was excised from the agarose gel with a clean, sharp scalpel or by using an x-tracta agarose gel extraction tool (LabGadget, USA, LG202). Purification of DNA from agarose gel was carried out using the QIAquick® gel extraction kit (Qiagen, USA, cat no. 28704). The gel slice was weighed and 3 volumes of Buffer QB to 1 volume of gel (100 mg ~ 100 µl) was added. This was incubated at 50°C for 10 minutes (or until the gel slice had completed dissolved). To help dissolve the gel, the tube was mixed by vortexing every 2 - 3 minutes during the incubation. After the gel slice had dissolved completely, the colour of the mixture was checked and had to be yellow (similar to Buffer QG without dissolved agarose). In cases were the colour was orange or violet 10 µl of 3 M sodium acetate, pH 5.0, was added to the mixture. One gel volume of isopropanol was then added to the sample and mixed. The sample was applied to a QIAquick spin column and centrifuged for 1 minute at 13,000 rpm to allow DNA to bind to the column. The flow through was discarded and 0.5 ml of Buffer QG was added to the column and

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centrifuged for 1 minute at 13,000 rpm. The column was washed by adding 0.75 ml of Buffer PE and the column left to stand for 5 minutes and then centrifuged for 1 minute at 13,000 rpm. The flow through was discarded and the column centrifuged again for 1 minute at 13,000 rpm. DNA was eluted by adding 30 µl of distilled water to the centre of the QIAquick membrane, the column was allowed to stand for 5 minutes and centrifuged for 1 minute at 13,000 rpm. Another 20 µl of distilled water was added to the centre of the QIAquick membrane, the column was allowed to stand for 5 minutes and centrifuged for 1 minute at 13,000 rpm bringing the total volume of DNA eluate to 50 µl.

Five µl of the gel purified PCR product was electrophoresed on a 1.5% (w/v) agarose gel in 1 X TAE buffer containing 10 mg/ml ethidium bromide (Sigma, cat no. E-1510) and viewed under UV illumination. The purified PCR product was quantified using the GENE QUANT *pro* (Amersham Biosciences, 89761) or NANODROP (ND 1000) spectrometer and the remainder of the gel purified product was stored at –20°C until needed.

C.6 Cloning of PCR products

Purified PCR products were cloned using the Qiagen PCR cloning kit (Qiagen, USA, cat no. 231124) which takes advantage of the single A overhang at each end of PCR products generated using *Taq* and other non-proofreading DNA polymerases. Five µl of ligation master mix, 50 ng of pDrive cloning vector and 40 - 60 ng of PCR product

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was mixed on ice to a final volume of 10 µl and the reaction mixture was incubated at 12°C for 2 hours in a thermal cycler. Transformation was carried out by adding 2 µl ligation-reaction mixture to 50 µl of high efficiency JM109 bacterial cells (Promega, USA, cat no. L2001) and the mixture mixed gently. It was incubated on ice for 5 minutes, at 42°C for 30 seconds and then placed on ice for 2 minutes. To the mixture, 250 µl of room temperature SOC medium (2 g/100ml Bacto tryptone, 0.5 g/100ml Bacto yeast extract, 1 M NaCl, 1 M KCl, 2 M glucose and 2 M Mg^{2+}) was added. Hundred µl, 50 µl and 20 µl of the transformation mixture was plated into Luria-Bertani (LB) (10 g/l Bacto tryptone, 5 g/l Bacto yeast extract, 5 g/l NaCl and 15 g/l agar) agar plates containing a final concentration of 100 µg/ml ampicillin supplemented with 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 80 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). The plate was incubated at room temperature until the transformation mixture had absorbed into the agar (~ 15 minutes), the plates were inverted and incubated at 37°C overnight (15 - 18 hours). A second incubation of the plates at 4°C for a few hours was carried out to enhance blue colour development and thereby facilitate differentiation between blue colonies and white colonies.

C.7 Screening of PCR products

White/blue colonies were screened for insert size by picking isolated individual clones and carrying out PCR using the T7 and SP6 primers (see Chapter 4 for primer sequence). For each 25 µl PCR reaction, 10 X PCR buffer, 3.3 µM of each primer

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and 1.5 mM MgCl2 and one unit of Takara *Taq* DNA polymerase (Takara Bio Inc. Japan, code R001AM), was used. Amplification was performed at initial denaturation of 94°C for 1 minute, 32 cycles of 94°C for 1 minute, 55°C for 30 seconds and 72°C for 30 seconds with a final extension step of 72°C for 10 minutes. Five µl of the PCR product was electrophoresed on a 1.5% (w/v) agarose gel in 1 X TAE buffer containing 10 mg/ml ethidium bromide (Sigma, cat no. E-1510) and viewed under UV illumination. Clones that contained the right insert size were grown on LB/ampicillin plates at 37°C overnight and plasmids were purified from them using the Qiagen plasmid mini kit as described below.

C.8 Plasmid purification

Plasmids were prepared from clones using the Qiagen plasmid mini Kit (Qiagen, USA. cat no. 12125). A single colony was picked and inoculated in 3 ml LB medium and incubated at 37°C overnight with vigorous shaking (200 rpm). Following this, the bacterial cells were harvested by centrifugation at 1,000 rpm for 15 minutes at 4°C. Bacterial pellets were resuspended by vortexing or mixing up and down in 0.3 ml of Buffer P1 (containing lyseblue reagent) until no cell clumps remained. Buffer P2 (0.3 ml) was added to cells and mixed gently to get a homogeneously blue coloured suspension. The mixture was incubated at room temperature for 5 minutes and 0.3 ml of chilled Buffer P3 was added and mixed immediately until all trace of blue had gone and the suspension was colourless. This was incubated on ice for 5 minutes and centrifuged at 13,000 rpm for 10 minutes. The supernatant was applied to a Qiagen-

tip 20 column equilibrated with 1 ml Buffer QBT and allowed to enter the resin by gravity flow. The column was washed two times with 2 ml Buffer QC and the DNA was eluted with 0.8 ml Buffer QF. DNA was precipitated with 0.56 ml room temperature isopropanol per 0.8 ml elution volume and centrifuged immediately at 13,000 rpm for 30 minutes. The DNA pellet was washed in 1 ml 70% ethanol, air-dried for 5 minutes and the DNA pellet was resuspended in 50 µl of distilled water. Two µl of the plasmid was electrophoresed on a 1% (w/v) agarose gel in 1 X TAE buffer (containing 10 mg/ml ethidium bromide (Sigma, cat no. E-1510) and visualised by UV illumination. Plasmids were quantified using the GENE QUANT *pro* (Amersham Biosciences, 89761) or NANODROP (ND 1000) spectrometer and stored at -20°C until needed.

C.8 Storage of plasmids

Plasmids were stored as glycerol stocks by adding 500 µl of autoclaved 30% glycerol to 500 µl of overnight culture. The mixture was vortexed briefly and stored at -70^{o}C. To recover the plasmid, the surface of the frozen culture was scraped using a sterile pipette tip and dipped into 3 ml of LB media and incubated overnight at 37°C with shaking at 200 rpm. The plasmid was purified using the method described previously.

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C.10 Sequencing of PCR products

PCR products were sequenced using the BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA, part no. 4337455).

C.10.1 Cycle sequencing

Cycle sequencing was carried out with the BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA, part no. 4337455). Each sample was sequenced using forward and reverse primers. A positive control DNA sample was included. For each sample, ready reaction mix (2.5X), BigDye Terminator v1.1/3.1 sequencing Buffer (5X), 3.2 pmol primer, ~ 40 ng of the PCR product was used in a 20 µl final volume. The positive control reaction consisted of the pGEM®-3Zf(+) double-stranded DNA control template and –21 M13 control primer (forward) (5'-TGT AAA ACG ACG GCC AGT -3').

Cycle sequencing was carried out by an initial denaturation of rapid thermal ramp to 96°C followed by 96°C for 1 minute, 25 cycles of rapid thermal ramp to 96°C, 96°C for 10 seconds, rapid thermal ramp to 50°C, 50°C for 15 seconds, rapid thermal ramp to 60°C, 60°C for 4 minutes and rapid thermal ramp to 4°C. Rapid thermal ramp was at 1°C /seconds. The content of the microcentrifuge tube was centrifuged briefly and prepared for extension.

C.10.1.1 Preparation of extension products

Sodium dodecyl sulfate (SDS)/heat treatment was carried out before the spin column purification to effectively eliminate unincorporated dye terminators from cycle sequencing reactions. To prepare extension products, 2 µl of 2.2% (w/v) SDS solution was added to each 20 µl completed cycle sequencing reaction. The tubes were sealed, mixed thoroughly and heated at 98°C for 5 minutes and cooled to 25°C for 10 minutes in a thermal cycler.

Extension products were purified using either the CENTRRISEP spin column (Princeton Separations, USA. CS-901) or the DyeEx 2.0 spin kit (Qiagen, USA, cat no. 63204) as described below.

C.10.2 Spin column purification

The products were purified using the CENTRISEP spin column (Princeton Separations, USA, CS-901). The columns were hydrated by reconstituting the gel in 0.80 ml of distilled water and vortexing briefly. Reconstituted columns were incubated at room temperature for 30 minutes before use. Air bubbles were removed from the columns by sharply tapping the sides. After the gel had settled and was free of bubbles, the top column cap and column end stopper was removed and excess

column fluid was allowed to drain into a 2 ml wash tube. Each column and wash tube was spun at 3,000 rpm for 2 minutes in order to remove interstitial fluid.

Samples were processed immediately by transferring 20 µl of completed DyeDeoxy terminator reaction mixture to the centre of the gel. The columns were then placed into a 1.5 ml sample collection tube and centrifuged at 3,000 rpm for 2 minutes. The purified samples were dried in a vacuum centrifuge for 10 - 15 minutes.

C.10.3 DyeEx 2.0 spin column purification

The products were purified using the DyeEx 2.0 spin column (Qiagen, USA, cat no. 63204). The spin column was gently vortexed to resuspend the resin. The cap of the spin column was loosened and the bottom closure of the spin column snapped off and placed in the spin column. This was then centrifuged for 3 minutes at 2,800 rpm. The spin column was transferred to a clean centrifuge tube and the sequencing reaction was applied to the centre of the gel bed and centrifuged for 3 minutes at 2,800 rpm. The eluate containing the purified DNA was dried in a vacuum centrifuge (Eppendorf concentrator 5301, Germany) for 10 - 15 minutes and stored at -20°C until needed for sequencing.

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C.10.4 Preparing and loading samples for capillary electrophoresis

Fifteen µl of TSR (Template Suppression Reagent) buffer was added to each sample pellet. This was mixed thoroughly on a vortex mixer and spun briefly in a microcentrifuge. Denaturation was carried out at 95°C for 2 minutes and immediately placed on ice for 1 minute. Samples were vortexed thoroughly, spun and transferred to 0.5 ml sample tubes and covered with tube septa for sequencing. The samples were loaded into a Spectrumedix SCE2410 genetic analyzer (SpectruMedix LLC, Pennsylvania, USA) using protocols recommended by the manufacturer.

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APPENDIX D

STANDARD PROCEDURES FOR BIOCHEMICAL ASSAYS

D.1 Biochemical analysis

Mosquitoes were individually homogenized on ice in 200 µl of distilled water and all the subsequent biochemical assays were performed on ice. Each microtitre plate included a sample of 10 individuals from the susceptible FANG strain. Two replicates of 10 µl aliquots of the homogenate were transferred to different microtitre plates for the GST and protein assays. For the monooxygenase assay, 20 µl of each homogenates were transferred to a separate microtitre plate. For the esterase assays, two 20 µl replicates of each mosquito homogenate were transferred to a separate microtitre plate while two 25 µl replicates of each homogenate were transferred to a separate plate for the acetylcholinesterase assay. Details of each assay are presented below.

D.1.1 Glutathione-S-transferase (GST) assay

Resistance mechanism by elevated GST can be detected by measuring GST activity for individual mosquitoes using chorodinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. Two hundred µl of GSH/CDNB working solution

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(10 mM GSH in 0.1 M phosphate buffer pH 6.5 and 63 mM CDNB diluted in methanol) were added to each replicate. Two blanks were included in each plate and contained 10 µl dH_2O and 200 µl of GSH/CDNB working solution. GST activity was measured kinetically at 340 nm for 5 minutes.

D.1.2 Monooxygenase assay

This assay measures the total haem cytochrome P450 content in individual mosquitoes. Eighty µl of 0.625 M potassium phosphate buffer pH 7.2 were added to the aliquots of the homogenates in each well followed by the addition of 200 µl of a tetramethyl benzidene solution (0.01 g 3,3',5,5' -tetramethyl benzidene diluted in 5 ml absolute methanol and mixed with 15 ml 0.25 M sodium acetate buffer pH 5.0). Twenty five µl of 3% hydrogen peroxide was added to each replicate and the mixture was incubated for 2 hours at room temperature. The controls contained 20 µl of distilled water and all the reagents excluding the homogenate. Absorbance was read at 650 nm.

D.1.3 Esterase assays

The esterase assays measures non-specific esterase activity directly using α and β naphthyl acetate as substrates on the same plate. Two hundred µl of α-naphthyl acetate solution (100 µl of 30 mM α-naphthyl acetate in acetone diluted in 10 ml of 0.02 M phosphate buffer pH 7.2) was added to one replicate and 200 µl of β-naphthyl

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acetate solution (prepared as for α-naphthyl acetate) was added to the other homogenate replicate. The enzyme reaction was ran for 30 minutes at room temperature followed by the addition of 50 µl of Fast blue stain solution (22.5 mg Fast blue in 2.25 ml distilled water, then 5.25 ml of 5% sodium dodecyl sulphate diluted in 0.1 M phosphate buffer, pH 7.0) to each well to stop the reaction. The controls contained 20 µl distilled water and all the reagents excluding the homogenate. Enzyme activity was read at 570 nm as an end point.

D.1.4 Altered acetylcholinesterase assay

Two 25 µl replicates of crude homogenate were solubilized in 145 µl Triton phosphate buffer (1% Triton X-100 in 0.1 M phosphate buffer pH 7.8). Ten µl of DTNB solution (0.01 M dithiobis 2-nitrobenzoic acid in 0.1 M phosphate buffer pH 7.0) and 25 µl of the substrate ASCHI (0.01 M acetylthiocholine iodide) were added to the first replicate of each homogenate. The latter solution was substituted by 25 µl of the substrate ASCHI containing 0.2% of the inhibitor propoxur (0.1 M) for the second replicate. Two control wells were included and contained 25 µl distilled water and all reagents with and without propoxur, respectively. The enzyme reaction was monitored by OD readings taken continuously at 405 nm for 5 minutes in a Kinetic Multiskan RC microtitre plate reader. The percentage of acetylcholinesterase inhibition by propoxur was calculated for each individual by comparing OD readings for uninhibited wells to inhibited wells.

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D.1.5 Protein assay

Protein concentrations for each mosquito (used as indicators of mosquito size) were used in the calculation of adjustments for total protein content, in order to make direct comparisons between OD values possible. Three hundred µl of BIO Rad protein reagent solution, prepared as a 1:4 dilution in distilled water, were added to 10 µl of each homogenate. The controls included 10 µl of distilled water and 300 µl of BIO Rad solution. The reaction was run for 5 minutes after which the OD of the reaction was read at 570 nm.

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