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The effect of environmental and metabolic stress on the expression of insecticide resistance phenotype and longevity in the Southern African malaria vectors *Anopheles arabiensis* and *Anopheles funestus*

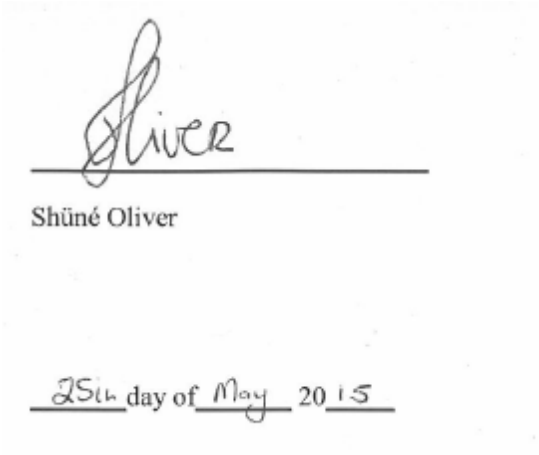
Shüné Oliver

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

Johannesburg, 2015

Declaration

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



Shüné Oliver

25th day of May 2015

This study would not have happened if my most beloved mosquito colonies were not as interesting as they were: SENN and SENN DDT, you will fascinate me always.

I would not have worked with these colonies if I had not been lured down the yellow brick road of Science by a curiosity and questioning sparked by my dearest Grandfather, John Kerspuy. This work is a testament to you.

“Mosquitoes remind us that we are not as high up on the food chain as we think”

~Tom Wilson

Abstract

Anopheles arabiensis and *Anopheles funestus* are the two dominant malaria vector species of Southern Africa. Insecticide resistance in these two species plays a major role in complicating malaria control efforts in the region. This study aimed to determine the role of environmental and metabolic stress on the insecticide resistance phenotype, development and longevity of malaria vectors. Furthermore, it aimed to whether the insecticide resistance phenotype affected the vector's capacity to cope with environmental stress, as well as examining the biochemical mechanisms that underpin these responses. Two laboratory *An. arabiensis* strains from Sudan were used: SENN, an unselected strain displaying baseline insecticide resistance and SENN DDT, a strain selected for DDT resistance from SENN. The unselected and permethrin-selected *An. funestus* strains FUMOZ and FUMOZ R were used, with the fully insecticide susceptible FANG strain used as a baseline control. The insecticide resistance phenotype, as well as a synergist and enzyme activity profile of all the strains were determined. Three environmental and metabolic stresses were examined: larval nutritional stress, oxidative stress and multiple bloodmeals. SENN DDT was found to be resistant to multiple classes of insecticide, with the phenotype mediated by both the L1014F *kdr* mutation as well as elevated detoxification enzyme activity. Pyrethroid and bendiocarb resistance in the FUMOZ R strain was confirmed to be metabolically mediated. Larval nutrient deprivation in *An. arabiensis* resulted in an increased developmental time and smaller adults with an increased susceptibility to DDT. In the SENN strain increased DDT susceptibility was found to be due to reduced vigour tolerance, while in the SENN DDT strain, the effect was due to significantly reduced metabolic enzyme activity. Insecticide resistant *An. arabiensis* and *An. funestus* strains were found to have a higher tolerance for oxidative stress, with this effect being mediated by significantly higher catalase and glutathione peroxidase enzyme activities in these strains. As all classes of insecticides were found to induce oxidative stress, it was hypothesised that this increased capacity to cope with oxidative stress was due to the requirement of coping with xenobiotic-derived reactive oxygen species. Synergism of oxidative stress enzymes had an

effect of the pyrethroid resistance phenotype, with synergism of the enzyme catalase strongly negating the pyrethroid phenotype of SENN DDT and returning the FUMOZ R strain to full pyrethoid susceptibility. Multiple blood feeding sustained the DDT and pyrethroid resistance phenotype of SENN DDT for up to 21 days. Multiple bloodmeals also had a greater effect of the longevity of the SENN DDT strain. These effects appeared to be mediated by the sustenance of the activity of Glutathione S-transferases with peroxidises in the SENN DDT strain. The blood component insulin significantly reduced the longevity of the SENN, but not the SENN DDT strain. Insulin supplementation also augmented the DDT resistance phenotype of SENN DDT in young females. This study therefore determined that that environmental stress affected both the life history and insecticide resistance phenotype of two major malaria vectors. Furthermore, resistant individuals appeared to cope better with the stresses, with the only marked fitness cost was reduced longevity in the SENN DDT strain. The study highlighted the importance of common environmental stressors on life history characteristics of importance for vector control, as well as the complicating role of insecticide resistance in the biology of vector species.

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List off abbreviations/acronyms

Ach	Acetylcholine
AchE	Acetylcholinestesterase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATZ	3-Amino-1, 2, 4-triazole
BSA	Bovine serum albumen
CDNB	1-Chloro-2,4-dinitrobenzene
DALY	Disability adjusted life years
DDT	Dichlorodiphenyl trichloroethane
DEM	Diethyl maleate
DNA	Deoxyribonucleic acid
DNPH	Dinitrophenyl hydrazine
DTNB	dithiobis 2-nitrobenzoic acid
DTT	Dithiotreitol
DVS	Dominant vector species
EDTA	Ethylenediamine tetracetic acid
ER	Endoplasmic Reticulum
GABA	γ-Amino butyric acid
GPOX	Glutathione peroxidase
GSH	Reduced Glutathione
GSSG	Oxidised Glutathione
GST	Glutathione S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ILP	Insulin-Like peptide
IRAC	Insecticide resistance action committee
IRS	Indoor Residual Spraying
kdr	Knock down resistance
LC	Lethal concentration
LD	Lethal dose
LLINs	Long lasting insecticide treated nets
MFO	Mixed function oxidases
MFO	Mixed Function Oxidases
NADH	Nicotinamide adenine dinucleotide phosphate
OP	Organophosphate
PBO	Piperonyl butoxide
PMSF	Phenyl methyl sulfonyl fluoride
PUFA	Polyunsaturated fatty acids
rdl	Resistance to dieldrin locus
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SBC	SENN base control
SBF	SENN base fasted
SDC	SENN DDT control
SDF	SENN DDT fasted
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TGF	Transforming growth factor
TMB	3,39,5,59-tetramethyl benzidine
TPP	Triphenyl phosphate
Vg	Vitellogenin
WHO	World Health Organisation

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Chapter 1: Introduction, Literature Review and Common Methodologies

1.1 The burden of vector borne diseases

The order Diptera is of particular importance to medical entomologists due to the major role certain species in this order play in the global burden of disease. Several of the world's most important communicable diseases have in common the requirement of an intermediate host. Such diseases are referred to as vector borne diseases (Hill *et al*., 2005).

The major vector borne diseases of humans can be divided into diseases caused by protozoans (Malaria-*Plasmodium* spp., Leishmaniasis-*Leishmania* spp., Human African Trypanosomiasis-*Trypanasoma brucei*, Chagas diseases-*Trypanasoma cruzi*); arboviral diseases such as Dengue fever and Yellow fever; and filarial nematode diseases such as lymphatic filariasis, onchocerciasis and *Loa loa*. With the exception of Chagas disease, which are spread by *Triatoma* species (hemiptera), all the aforementioned are transmitted by biting flies (Hill *et al*., 2005, Lane and Crosskey, 1993). Of the biting flies, mosquitoes are probably the most important vectors of human disease, rivalled only in importance and variety by ticks, which transmit at least 14 diseases, including babesiosis, Lyme diseases, Rocky Mountain spotted fever and Congo-Crimean haemorrhagic fever (Lane and Crosskey, 1993).

Malaria is the most important of the vector borne diseases (Breman and Brandling-Bennett, 2011). In the global burden of communicable diseases, it ranks as the third leading cause of death after HIV/AIDS and tuberculosis (WHO, 2013a). Mortality, however, is not the only measure of disease burden. Disability adjusted life years (DALYs) are defined as "one lost year of healthy life, and is a measurement of the gap between the current health of a population and an ideal situation where everyone in the population lives to an old age in full health" (Hill *et al*., 2005). In terms of DALYs the burden of malaria is greater than that of tuberculosis (Curtis and Davies, 2001, WHO, 2013b). Sub-Saharan Africa bears a disproportionate burden of malaria. Of the estimated 290 million cases of malaria in 2010, 90% of deaths occurred in Africa (WHO, 2013b). This may be attributed to many particular factors. The first involves the complexity of the triad of organisms involved in the

transmission of the disease. As the *Plasmodium* parasite has co-evolved with humans (Carter and Mendis, 2002) it is particularly well suited to evade the various attempts made to control it. It has a complex, AT rich genome, making it a difficult organism to work with (Oyola *et al*., 2012) and its high genetic variability due to a variety of mechanisms including alternative splicing, results in the rapid emergence of drug resistance (Hull and Dlamini, 2014). Accordingly, vaccine development has been particularly difficult, and it does not appear that a vaccine will be available in the near future (Vaughan and Kappe, 2012).

The human host also plays a role in the transmission of malaria. Malaria has driven the evolution of sickle cell anaemia, a condition that makes it more difficult for the parasite to infect the erythrocyte (Banuls *et al*., 2013). Malaria infection is also highly dependent on the host's immune response, and partial immunity is common in highly endemic areas (Offeddu *et al*., 2012) although immune-compromised persons, such as HIV patients, young children and pregnant women, are particularly vulnerable (Bates *et al*., 2004).

Perhaps the most difficult factor in the malaria infection triad is the vector. Vector control efforts are hampered by operational difficulties and environmental factors such as climate. This is largely where the African challenge comes in, as the vectorial system in Africa is particularly complex.

1.2 The complexity of the African malaria vector system

The genus *Anopheles* consists of a variety of species complexes comprised of isomorphic sibling species. It is estimated that "at least half of the important vectors of malaria belong to sibling (or cryptic) species" (Harbach, 2013). The challenge for mosquito biologists is that members of the same complex can vary greatly in behaviour, particularly in their refractoriness (resistance) to *Plasmodium* and their host preferences. As such, in the same complex anthropophilic mosquitoes that are excellent *Plasmodium* vectors can exist in sympatry with morphologically identical or highly isomorphic species which are zoophagic, refractory to the parasite, or both factors in combination (Dadzie *et al*., 2013). Furthermore, two vectors can exist in sympatry, but differ in their larval habitat or adult behaviour, meaning that a blanket vector control system cannot be implemented (Subbarao and Sharma, 1997, Russell *et al*., 2013). Therein lies the challenge of African malaria control- the most efficient

vectors of malaria reside on this continent and their systematics are particularly complex. This is highlighted in figure 1.1.

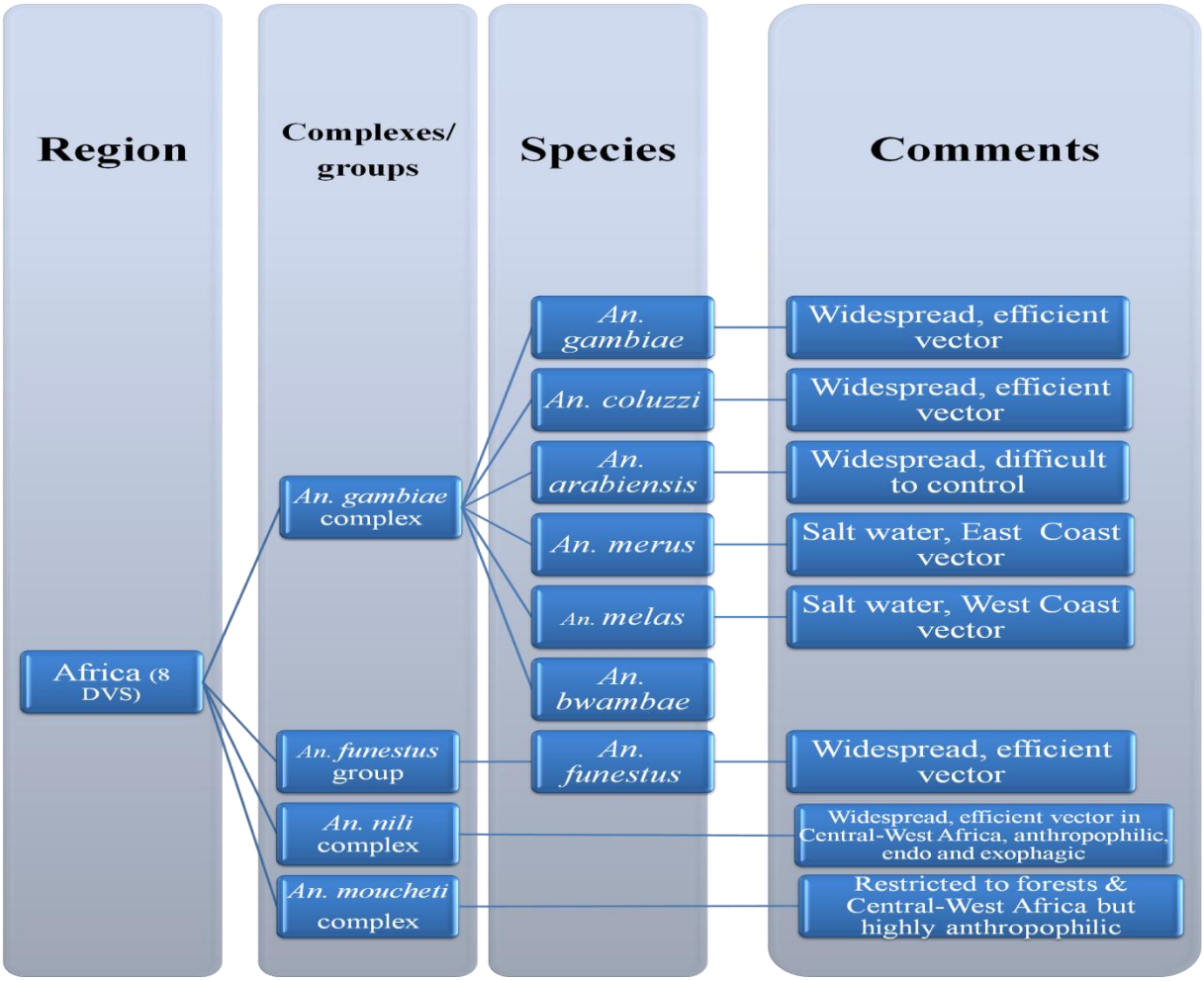


Figure 1.1: Composition of the dominant vector species (DVS) of Africa. Of the eight species recognised as Dominant Vector Species in Africa, 6 are members of the *An. gambiae* complex. The figure highlights the relationships between the DVS as well as the locality of each vector (Gillies and de Meillon, 1968, Sinka *et al*., 2010).

1.3 The vector *Anopheles arabiensis*

Often occurring in sympatry with *An. gambiae* and *An. coluzzii* is their sibling species, *An. arabiensis* (Sinka *et al*., 2010). This species is, however, not as well researched as its' siblings as it is not considered to be as efficient a malaria vector as *An. gambiae* and *An. coluzzii*. There has been evidence, though, that *An. arabiensis* can support a parasite load similar to *An. gambiae* and *An. coluzzii* (Gneme *et al*., 2013).

Like its' siblings, *An. arabiensis* breeds in temporary pools of shallow fresh water that are sunlit and without excessive vegetation (Gillies and Coetzee, 1987). They are, however, often found in areas with irrigated agricultural systems such rice growing areas (Mwangangi *et al*., 2006, Ye-Ebiyo *et al*., 2003). The significance of these larval habitats will be discussed in chapter 3. Unlike *An. gambiae*/*An. coluzzii, An. arabiensis* appears to be able to utilise a greater variety of larval habitats. These include slow flowing water, turbid, polluted and brackish water (Sinka, 2013, Sinka *et al*., 2010).

The bionomics of the adult mosquito is what distinguishes *An. arabiensis* from its' sibling species. This species is considered a primary vector in arid environments. It is found in dry environments, savannah and sparse woodlands (Gillies and de Meillon, 1968, Sinka *et al*., 2010). It is a very widespread species, ranging from the Sahel in the north to Namibia, Botswana and South Aftica in the south. Although the species is widely distributed, it is notably absent from the humid forested areas of West Africa (Sinka, 2013). They can, however, be found in forested areas where land has been cleared (Coetzee *et al*., 2000).

Anopheles arabiensis is extremely variable in biting and resting behaviour. It is both exo and endophagic and exhibits both endo-and exophily. This vector is highly adaptable, and displays great behavioural plasticity. It has been proposed that east African populations of *An. arabiensis* tend to be zoophilic and exophilic, while west African populations tend towards anthropophily, endophily and endophagy (Tirados *et al*., 2006). *Anopheles arabiensis* are also highly opportunistic, and have been reported to feed on cattle, chickens and goats (Mwangangi *et al*., 2003). Their behavioural plasticity contributes greatly to the difficulty in controlling this vector. Their tendency towards exophagy means that long-lasting insecticide treated nets (LLINs) are not as effective against this species (Kitau *et al*., 2012). Their exophilic tendencies (Sinka *et al*., 2010) mean that indoor residual spraying (IRS) is not as effective a control measure as for endophilic species such as *An. gambiae* and *An. funestus* (Sharp *et al*., 1990). Despite their zoophagy, they are often not diverted from humans by the presence of cattle. This means that zooprophylaxis is not truly feasible for vector control (Tirados *et al*., 2011). All these factors, in combination, make *An. arabiensis* a particular challenge for vector control efforts.

1.4 The *Anopheles funestus* group

Anopheles funestus is the nominal member of the *An. funestus* group. Members of the *funestus* sub-group, *An. funestus, An. funestus-like, An. parensis, An. vaneedeni* and *An. aruni*, are morphologically similar, while those in the *rivulorum* sub-group, *An. rivulorum*, *An. leesoni*, and *An. confusus*, can be distinguished from each other at different life stages. *Anopheles fuscivenosus* has distinct adult characteristics (Gillies and Coetzee, 1987, Gillies and de Meillon, 1968).

As in the *An. gambiae* complex, cytogenetic evidence suggests potential incipient speciation in the *An. funestus* population of Bukina Faso (Costantini *et al*., 1999, Dia *et al*., 2013, Guelbeogo *et al*., 2005). The two chromosomal forms are called "Folonzo" and "Kiribina". These two forms are found in sympatry in Senegal, with the Kiribina form being more abundant, and having higher biting rates, and are therefore responsible for the bulk of malaria transmission (Dia *et al*., 2013).

After the discovery of the Kiribina and Folonzo chromosomal forms, *An. funestus* s.s. was also divided into three molecular types. The M-form is found in East Africa, the W-form in West Africa and the MW-form in Southern Africa (Garros *et al*., 2004). This situation is further complicated by atypical forms found in Tanzania and Kenya, as well as the discovery of a Type Y and Type Z (Koekemoer *et al*., 2006). The complexity within the *An. funestus* vector itself suggests the existence of an *An. funestus* complex within the Funestus group (Sinka *et al*., 2010).

New species within the Funestus and Rivulorum sub-groups are still being discovered. A new species of *An. rivulorum* was reported in Cameroon in 2003 (Cohuet *et al*., 2003), as was a new species of *An. funestus* in Malawi in 2009 (Spillings *et al*., 2009). The Cameroonian and Malawian species are referred to as *An. rivulorum*-like and *An. funestus*-like respectively. Mitochondrial DNA analysis has also demonstrated the existence of two distinct clades within *An. funestus* southern Africa. This may suggest a speciation event (Michel *et al*., 2005, Choi *et al*., 2014) although this needs to be further investigated.

Anopheles funestus is the only member of the Funestus sub-group directly implicated in malaria transmission (Gillies and De Meillon, 1968). *Anopheles rivulorum* has twice been implicated in malaria transmission in Tanzania (Wilkes *et al*., 1996) and *An. vaneedeni* can be infected with parasites in a laboratory setting (De Meillon *et al*., 1977).

Like *An. gambiae*, *An. funestus* is an extremely efficient vector of malaria, due to its strongly anthropophagic and endophilic tendencies (Gillies and Coetzee, 1987, Gillies and de Meillon, 1968, Sinka *et al*., 2010).

Unlike members of the *An. gambiae* complex, members of the Funestus group breed in permanent or semi-permanent, well vegetated bodies of water, of natural or artificial origin. They have a preference for floating vegetation over emerging vegetation (Gillies and de Meillon, 1968).

Anopheles funestus displays relatively consistent behaviour compared to other African vector species, with marked anthropophagy and endophily. Mosquitoes displaying these types of behaviours make effective targets for indoor residual spraying (Russell *et al*., 2013). Although endophilic anthropophily is the norm for this vector, it is not the sole exhibited behaviour and *An. funestus* is a remarkably adaptive mosquito. Of particular interest is an extreme example of shift in biting time observed in Senegal, where six times more *An. funestus* were captured biting during the early evening than during the night (Sougoufara *et al.*, 2014).

During 1999/2000 had its' worst malaria epidemic in 50 years. Although the situation was complicated by drug resistance in the parasite, the majority of the blame for this epidemic was placed on the re-emergence of insecticide resistant *An. funestus* (Hargreaves *et al*., 2000). South Africa had changed its IRS regime from DDT (Dichlorodiphenyl trichloroethane) to the pyrethroid deltamethrin in 1996, unware that a pyrethroid resistant population of *An. funestus* was present in Southern Mozambique (Maharaj *et al*., 2005). The significance of this population of *An. funestus* will be further expanded in chapter 2.

1.5 What makes a good vector?

Not all species of anophelines are malaria vectors. An obvious requirement is non-refractoriness to *Plasmodium*, the ability to allow the parasite to complete its' life cycle within the mosquito host, essentially an immunocompromise in the mosquito (Dimopoulos *et al*., 2001, Sinden *et al*., 2004).

Yet this is not the sole determining factor in what makes a mosquito a successful vector. These factors can be divided into two broad categories. The first is how well the female performs in obtaining her blood meal. This includes host seeking and frequency of blood feeding. The second category involves survival and reproduction. These aspects include factors such as fertility, fecundity and longevity. These factors are referred to as life history parameters and are important determinants of an organisms' fitness (Grech *et al*., 2007). Development time is important as the longer it takes larvae to develop, the likelihood of reaching adulthood is reduced due to the potential of predation and human interference (Padmanabha *et al*., 2011). Longevity is a crucial determinant of vector competence in a disease such as malaria, which has an intrinsic incubation period. If the mosquito does not live long enough for the parasite to be transmitted, the mosquito's vector competence is compromised. As such, small changes in longevity have a significant impact on disease transmission (Garrett-Jones and Shidrawi, 1969).

Factors such as fertility and fecundity were avoided in this study, as evidence suggests that conclusions about life history traits in laboratory strains are often not applicable to wild populations (Baker, 1964, Huho *et al*., 2007). Although not a life history characteristic, a crucial determinant of vector competence is insecticide resistance. Insecticide resistance is a complex factor, as it will affect the aforementioned fitness traits. Fitness costs of insecticide resistance are well researched in *Culex* (Bourguet *et al*., 2004, Kliot and Ghanim, 2012, Rivero *et al*., 2010). The most cited study of fitness costs in *Anopheles* is a study of fitness costs associated with resistance in *An. funestus*. Unlike in *Culex*, pyrethroid resistance did not appear to incur a fitness cost (Okoye *et al*., 2007). Therefore, if factors determining vector competence can be broadly categorised into blood feeding behaviours and factors affecting

post blood meal survival, insecticide resistance must be considered an important determinant of vector competence.

1.6 Insecticide resistance: The scale of the problem

The World Health Organisation (WHO) defines insecticide resistance as "the developed ability in a strain of insects to tolerate doses of insecticide that would prove lethal to the majority of individuals in a normal population of the same species" (WHO, 1957). From an operational perspective, insecticide resistance can be defined as the "heritable change in the sensitivity of a population to an insecticide, which is reflected in the repeated field failure of that product to achieve the expected level of control when used according to the label recommendation of that pest species, and where problems of product storage, application and unusual climactic or environmental conditions can be eliminated" (McCaffery and Nauen, 2006).

Insecticide resistance is a growing problem in all pests that affect public health, including mosquitoes, cockroaches, bedbugs, houseflies, fleas and ticks (Nauen, 2007). Insecticide resistance is common in both *An. arabiensis* and *An. funestus*. *Anopheles funestus* resistance hotspots include South Africa, Mozambique, Kenya and various regions of West Africa. Insecticide resistance is common in *An. arabiensis* populations, with hotspots including Kenya and Ethiopia (www.IRmapper.com) (Knox *et al*., 2014). The importance of insecticide resistance is indicated by the sharp increase in numbers of publications on the subject, as this reflects funding and interest in the subject. The existence of the Insecticide Resistance Action Committee (IRAC) also highlights the problem (Hardy, 2014).One of the primary functions IRAC has performed is to classify insecticides according to their mode of action. Although various classes of insecticides exist, only four classes of adulticidal insecticides are available for public health usage (Nauen, 2007). Their IRAC categories are summarised in table 1.1.

Table 1.1: IRAC classifications of insecticides used for mosquito control. Adulticides are highlighted in yellow, larvicides are highlighted in blue.

Action	Class	Insecticides
Acetylcholinesterase inhibitors (Nerve action)	1A	Carbamates
	1B	Organophosphates (OPs)
GABA gated chloride antagonists (Nerve action)	2A	Organochlorines, cyclodienes
	2B	Phenyl parazoles* (not used for mosquitoes)
Sodium Channel modulators	3A	Pyrethroids/Pyrethrins
(Nerve action)	3B	DDT/Methoxychlor
Juvenile Hormone Mimics	7A	Juveneline hormone mimics
	7C	Pyripoxifen
Acetylcholinesterase inhibitors (Nerve action)	1B	Organophosphates
	5	Spinosyns
Chitin biosynthesis inhibitors	15	Diflibenzuron, Novaluron
Microbial disruptors of insect midgut	11	*Bacillus thuringensis* toxin

As adult mosquito control is primarily neurotoxic, targeting only two sites, it is not surprising that a modification of the target site can result in resistance to unrelated chemical compounds. This phenomenon is known as cross resistance (Brogdon and McAllister, 2004). Cross resistance and a lack of new insecticides available for public health use has been greatly exacerbated the problem of insecticide resistance (Zaim and Guillet, 2002). Although various adulticidal compounds have been identified from different IRAC classes commonly used for agriculture (Paul *et al.*, 2006), the transition to availability for public health usage has not yet occurred. A notable exception is the class 21 mitochondrial uncoupler chlorphenapyr (Black *et al.*, 1994) which has shown promise as an adulticide against various species of mosquitoes both in the laboratory setting as well as the field (Oliver *et al.*, 2010, Oxborough *et al.*, 2010).

Insecticide resistance is a major challenge for the control of vector borne disease, and as such, a thorough understanding of the biological basis of this phenotype is crucial. The broadest aim of the study was to contribute to the elucidation of the biological mechanisms underpinning resistance. To understand this, it is necessary to understand the current knowledge of insecticide resistance.

1.7 Mechanisms of insecticide resistance

Mechanisms of resistance can be divided into two categories: mechanisms to decrease the effective dose of the insecticide, and mechanisms to render the dose of insecticide ineffective (Ranson *et al*., 2002). This is summarised in figure 1.2:

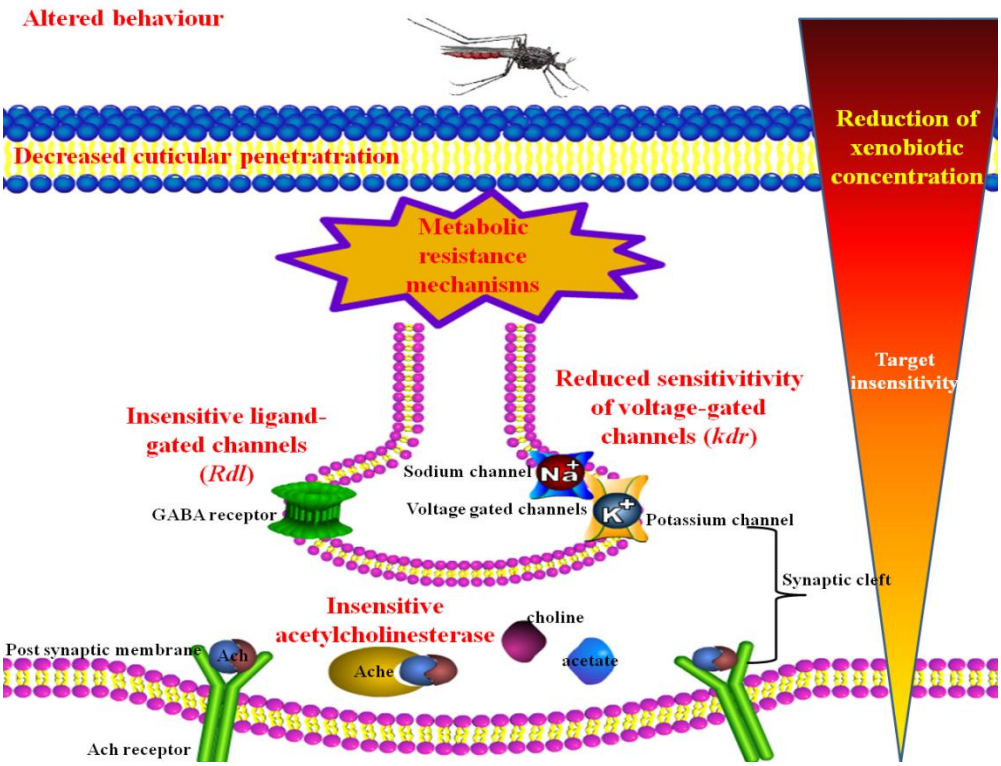


Figure 1.2: Generalised scheme of mechanisms employed by mosquitoes to produce a resistance phenotype. All mechanisms are highlighted in red text. The mechanisms corresponding to the red region on the concentration gradient (altered behaviour, decreased cuticular penetration and metabolic mechanisms) are all targeted to reducing the effective concentration. The remaining mechanisms, all resulting in insensitivity of the target site to the xenobiotic, are all aimed at rendering the dose of the insecticide inactive. (Based on Ffrench-Constant *et al*., 1998, Li *et al*., 2007, Ranson *et al*., 2002).

1.7.1 Reduction of effective dose

In the broad sense, there are four ways of coping with a xenobiotic: avoidance of threat, impeding the entry of the xenobiotic, increased metabolism of the xenobiotic, and finally preventing it from interacting with its' intended target site. Before various complex metabolic systems come into play, simple behavioural avoidance mechanisms can be employed. Behavioural avoidance can involve the reduction of entry into houses, and a shift in biting times (Hemingway *et al*., 2004). *Anopheles arabiensis* displays extensive behavioural plasticity and tends to shift in biting times. As they also display an even greater tendency towards exophagy and exophily this vector is extremely difficult to control (Ferguson *et al*., 2010, Govella *et al*., 2013). *Anopheles funestus* has also been demonstrated to have shifted in biting times after the implementation of long lasting insecticide treated nets (LLINs) (Sougoufara *et al*., 2014). In the villages of Lokohoué and Tokoli, Benin, even though no deltamethrin resistance was found, the vectors changed their behaviour after the introduction of LLINs. Biting times increased to later in the morning, and the percentage of outdoor biting increased in this normally endophilic species (Moiroux *et al*., 2012). The excito-repellency properties of certain insecticides, particularly pyrethroids, the mainstay of LLINS, can also induce avoidance behaviour (Potikasikorn *et al*., 2005, Sungvornyothin *et al*., 2001). Behavioural changes are therefore difficult to monitor, but crucial mechanism of the effective dose.

The importance of the cuticle as a barrier to insecticides was a factor considered early in vector biology (Mukerji and Chatterjee, 1953, Webb and Green, 1945). Since then, thickened cuticles have been reported in various insects such as *Culex pipiens* (Stone and Brown, 1969), *Cx. tarsalis* (Apperson and Georghiou, 1975) and *An. funestus* (Wood *et al*., 2010). The hypothesis is that a thicker cuticle would reduce the penetration of the insecticide and as such would reduce the effective dose of the insecticide (Yu, 2008). None of the aforementioned studies speculate about the importance of penetration through the insect's spiracle, although this is a particular challenge to decreased penetration (Korsloot *et al*., 2004). Furthermore, insecticide penetration rates do not always differ in resistant strains, as no significant difference in permethrin penetration in an insecticide resistant versus susceptible *Aedes*

aegypti strain was noted (Kasai *et al*., 2014). Nevertheless, reduced insecticide penetration is an important mechanism of reducing the effective dose of the insecticide.

The final way of reducing the effective dose is by removing the xenobiotic before it can reach the target site. This is known as metabolic resistance, as the mechanism is mediated by increased metabolism of the pesticide. Pesticide metabolism occurs naturally in all insects, but in resistant individuals, the turnover of the pesticide is rapid enough so that the insecticide does not overwhelm the system. Metabolic detoxification is mediated by three enzyme superfamilies: the general esterases, the cytochrome P450s and the glutathione s-transferases (GSTs) (Ranson *et al*., 2002). The superfamilies consist of a number of duplicated genes that tend to be clustered together rather than being distributed throughout the genome. The continuous gene duplication within these superfamilies have allowed functional diversity. This is facilitated by the proteins' permissiveness to changes in their primary structure. These alterations result in broad scale changes in substrate specificity which helps to facilitate resistance (Ranson *et al*., 2002).

There are numerous mechanisms for increasing the avtivity of these enzymes. These include *cis* and *trans*-acting regulatory elements and amplification of the gene encoding the enzyme. Gene amplification is well described for esterase-based organophosphate resistance in *Culex pipiens*. Upregulation of cytochrome P450s activity has been attributed to both cis-and-trans-acting regulatory elements as well as gene duplication (Bass and Field, 2011). These three superfamilies need to be explored in more detail to understand their function and significance.

1.7.1.1 General Esterases

Esterases (E.C. 3.1.1) are a super family of proteins characterised by an α/β hydrolase fold that are capable of hydrolysing ester bonds (Oakeshott *et al*., 1993) and in dipterans they occur as a gene cluster on the same chromosome (Brogdon and McAllister, 2004). The active site of esterases has a characteristic nucleophilic catalytic triad with serine as the primary amino acid (Polgár, 2005). The most important of these enzymes in the context of insecticide resistance are members of the carboxylesterase gene family, which contain the carboxyl/cholinesterase. This family contains proteins with strong substrate selectivity, such as juvenile hormone

esterase and acetylcholinesterases, less specific esterases, and non catalytic proteins such as neurolignins and neurotactins (Montella *et al*., 2012).

Esterase nomenclature remains a difficult matter, with many researchers still using the system proposed by Aldridge (1953). This classification system is based on their interaction with organophosphates. Esterase-A (EstA) are capable of hydrolysing Organophasphates (OPs), Esterase-B (EstB) are inhibited by OPs, and Esterase C (EstC) do not interact with OPs (Aldridge, 1953a, Aldridge, 1953b). Although this classification is flawed due to the fact that not all esterases fit neatly into this category, it is still commonly used by vector biologists in favour of the system proposed by the International Union of Biochemistry and Molecular Biology (IUBMB), which names all enzymes by an E.C. number. According to this system, the esterases of importance are EC3.1.1.1; carboxylic ester hydrolases such as acetylcholinesterases, were previously known as aliesterases, EstB or carboxylic ester hydrolases which act on aliphatic molecules. Arylesterases, E.C. 3.1.1.2, were previously known as paraoxanases, EstA or aryl-ester hydrolases, which preferentially act on aromatic compounds (Montella *et al*., 2012).

Elevated esterase activity is a common mechanism of mediating resistance to carbamates and OPs in a range of pests. This enzyme family can produce either a broad or narrow spectrum of resistance. Broad spectrum resistance is achieved by sequestration of the insecticide, a rapid binding and slow turnover of the bound insecticide. Hydrolysis of insecticides containing ester bonds is also possible, but it produces a much narrower spectrum of resistance. As sequestration of compounds is less specific than hydrolysis of ester-containting compounds, sequestration results in resistance to more compounds than hydrolysis does (Karunaratne *et al*., 1995).

Esterase based resistance is best characterised in *Culex* mosquitoes and the peach potato aphid *Myzus persicae*. In *Culex* species where sequestration-mediated resistance occurs, esterases can constitute a significant proportion of the total soluble protein (Paton *et al*., 2000). In resistant *M. persicae* the esterases can make up 1% of the total mass of the insect (Heckel, 2012). This makes these enzymes excellent candidates for scavenging of xenobiotics. To achieve such high quantities of enzyme, the esterase genes are usually amplified (Mouches *et*

al., 1986). Gene amplification is the result of gene duplication, and as mentioned earlier, is a common occurrence in the members of three enzyme superfamilies associated with insecticide resistance (Ranson *et al*., 2002). Esterases which increase resistance by increased pesticide hydrolysis, in contrast, tend to be due to point mutations in the structural gene which can alter the substrate specificity of the enzyme (Hemingway, 2000). An example of this is a tryptophan to leucine substitution at position 251 in the E3 esterase *of Lucilia cuprina*. This mutation alone confers malathion resistance, while a glycine to asparagine mutation 137 confers broad spectrum resistance to a range of other OPs (Campbell *et al*., 1998). A malathion specific esterase without any increase in general esterase activity has been described in various mosquito species (Karunaratne and Hemingway, 2001). Malathion carboxylesterase is a particularly efficient enzyme (Whyard *et al*., 1994) It is thought that this activity of this malathion carboxylesterase is due to structural differences in this esterase (Hemingway and Karunaratne, 1998: Ziegler *et al*., 1987). In the brown planthopper *Nilaparvata lugens*, variations in esterase activity are mediated by post translational modification, such as differential glycosylation and phosphorylation (Small and Hemingway, 2000).

Esterases in *Culex* are very well characterised, but this is not true for *Anopheles* species. Besides the molecular and biochemical characterisation of these enzymes in *Culex*, the fitness cost of esterase based resistance has been examined in *Culex*, and it has been found that esterase-mediated resistance results in a reduction of energy reserves (Rivero *et al*., 2011). The most reported mechanism of esterase based resistance in *Anopheles* appears to be increased rate of metabolism (Hemingway and Ranson, 2000), but the fitness costs associated with esterase-mediated resistance has not been examined in this species.

1.7.1.2 Cytochrome P450s

The diversity of the super family of P450 enzymes is hinted at by the many names associated with these proteins. They are called mixed function oxygenases/oxidases, microsomal oxidases, haeme thiolate proteins, polysubstrate monoxygenases and cytochrome P450 momoxygenases (Feyereisen, 1999). In this study, this diverse group of haeme-containing

proteins will be referred to as cytochrome P450s, due to their distinctive absorbance spectrum at 450nm (Omura and Sato, 1964).

Cytochrome P450s (E.C. 1.14--) are ubiquitous enzymes found in all aerobic organisms, both prokaryotes and eukaryotes (Nelson *et al*., 1996) and have been found in viruses as well (Lamb *et al*., 2009). It is believed that the genome of many insect species carry at least 100 P450 genes which have evolved from a single common ancestral gene. This makes them one of the largest and oldest gene superfamilies (Feyereisen, 1999).

This enzyme family is involved in a diverse array of biological functions. They are involved in insect growth, development and reproduction due to their involvement in the biosynthesis of ecdysteroids and juvenile hormones. They are also involved in the degradation of odorants, pheromones and defensive chemicals, but most importantly for this study, the metabolism of xenobiotics (Feyereisen, 1999). As these enzymes can catalyse up to 60 different reactions, they have been referred to as diversozymes (Coon *et al*., 1996).

Due to the large number of these enzymes, a strict nomenclature is required. All gene members of the P450 family are referred to with prefix CYP, followed by a number for the family, a letter for the subfamily, and a number for the individual gene or gene product. Families share >40% amino acid sequence identity, subfamilies share >55% amino acid identity (Nebert *et al*., 1987, Nelson *et al*., 1996). Insect P450s are assigned to 6 CYP families, 5 of which are insect specific- CYP 6, 9, 12, 18 and 28. CYP4 includes sequences found in vertebrates (Feyereisen, 1999).

At a tissue level, P450s are found in the midgut and fat body, indicating that they are involved in detoxification (Scott and Lee, 1993). On a cellular level, mitochondrial and microsomal P450 systems have been described (Feyereisen, 1999). Although most interest in xenobiotic metabolism has focussed on microsomal P450s, it has now been demonstrated that there are multiple mitochondrial P450s capable of detoxification (Guzov *et al*., 1998). As P450s were found in the mitochondrial and microsomal fraction of the cell, it was easily discovered that unlike the other two superfamilies involved in detoxification, they are membrane bound, rather

than soluble proteins (Feyereisen, 1999, Kaewpa *et al*., 2007), making these proteins more difficult to work with than the other two superfamilies.

Some reactions result in bioactivation of compounds, while others result in detoxification of xenobiotics. Whether the effects of the cytochrome has a toxic result or results in detoxification depends on the nature of the initial P450 metabolite. Most stable metabolites are simply excreted. Where a toxic or reactive metabolite is formed, the product can either undergo further metabolism and be excreted, or it can reach its' target in its' reactive form and exert a toxic effect (Korsloot *et al*., 2004).

Some of the most important toxic reactive metabolites of the cytochrome P450 catalysis are reactive oxygen species (ROS). These enzymes are one of the greatest endogenous sources of ROS in the cell (Siraki *et al*., 2002). The significance of this will be discussed in greater detail in chapter 4.

P450 enzymes have been associated with resistance to every class of insecticide, including those not used for vector control, such as insect growth regulators (Bergé *et al*., 1998, Brogdon and McAllister, 2004). This has been demonstrated by the association of elevated transcript levels with resistance, as well as the loss of phenotype by inhibition of the enzyme system (synergy) with the "suicide inhibitor" piperonyl butoxide (PBO) Suicide inhibitors are so termed because they bind their substrates irreversibly and as such cannot serve any further function. (Feyereisen, 1999).

P450s have also been implicated in resistance by the direct demonstration of their ability to metabolise the insecticide of interest. Examples include CYP6A2 in *D. melanogaster* which can metabolize diazinon and cyclodienes (Dunkov *et al*., 1997), CYP6M2 can metabolise type I and II pyrethroids (Mitchell *et al*., 2012) and CYP6P9 in *An. funestus* which can metabolise pyrethroids (Riveron *et al*., 2013) . Interestingly, CYP6Z8 of *Aedes aegypti* and CYP6Z2 of *An. gambiae* can metabolise the hydrolysed products of carboxylesterases (Chandor-Proust *et al*., 2013). It is also interesting to note that altered P450 activities are also reported in conjunction with increased activities of other systems (Hemingway *et al*., 2004). The significance of this will be discussed later in this chapter.

Like esterases, increased P450 activity is commonly a result of gene amplification. As with the other two gene superfamilies these duplicated genes tend to cluster together with only 22 of the 111 P450 genes in *An. gambiae* appearing as single genes (Ranson *et al*., 2002). It is believed that gene duplication from the original ancestral P450 gene is responsible for these clusters (Feyereisen, 1999). The increased level of mRNA transcripts due to gene amplification-induced overexpression commonly underpin most P450 mediated resistance, although stabilisation of the mRNA transcripts cannot be ruled out (Bergé *et al*., 1998).

Overexpression of mRNA is not the only source of P450 mediated resistance. Like with esterases, single amino acid changes in the enzyme can change substrate specificity in these already catalytically plastic enzymes (Feyereisen, 1999). A good example of this is CYP6A1 of *Musca domestica*. Although resistance in the fly can be synergised by PBO, no oberservable increase in CYPA1 mRNA content could be observed, but increased catalysis could be attributed to three amino acid substitutions: R335S, L336V and V476Z. All these substitutions affect the active site of the enzyme (Bergé *et al*., 1998).

Another interesting case showing the importance of amino acid substitutions, as well as the complexity of P450 regulation is demonstrated in CYP6P9 of *An. funestus*. It was demonstrated that although mRNA levels and 7-ethoxycoumarin activity was elevated, that this was not mirrored by an increase in P450 protein expression (Stradi, 2011: unpublished). The increase in CYP6P9 catalytic efficiency in the resistant *An. funestus* strain appeared to be mediated by amino acid changes, which dramatically increased the substrate specificity and catalytic efficiency of the enzyme (Wondji *et al*., 2014).

Although cytochrome P450 enzymes are important in resistance to a number of insecticides, in this study, their involvement in pyrethroid resistance is of particular significance. This is because of their importance of mediating pyrethroid resistance in *An. funestus* populations in Southern Africa. This will be expanded upon in chapter 2.

1.7.1.3 Glutathione S-transferases

Glutathione S-transferases (GSTs), E.C. 2.5.1.18, belong to the last enzyme super family of major importance to insecticide resistance. Like esterases and cytochrome P450, they are a diverse family, and are characterised by the presence of a canonical thioredoxin fold (Martin, 1995). These ubiquitous, multifunctional enzymes, found in all aerobic organisms, catalyse the conjugation of reduced glutathione (GSH) onto their substrates by precipitating a nucleophilic attack on the electrophilic substrate (Chasseaud, 1979). The resultant metabolites, which were usually initially lipophilic, are then more water soluble than unconjugated substrates. Due to their newly introduced hydrophilic nature, these products are not likely to diffuse back through the lipid membrane (Habig *et al*., 1974). Certain GSTs are capable of using GSH as a cofactor rather than a conjugate to catalyse a dehydrochlorination reaction (Clark and Shamaan, 1984).

After the publication of the *An. gambiae* genome, it was remarked that the exact function of insect GSTs are unknown (Ranson *et al*., 2002). If homology from other organisms is taken into account, it can be concluded that GSTs serve solely as stress response proteins. Those stress responses can either be direct responses to stress, such as xenobiotic metabolism (Enayati *et al*., 2005), as a response to oxidative stress (Hayes and McLellan, 1999) or by mediating signalling cascades to cope with stress (Castro-Caldas *et al*., 2009, Gate *et al*., 2004). Therefore, despite their broad substrate specificity resulting in them being classified as multifunctional enzymes, unlike the other two superfamilies, which are involved in a wide array of biological activities, one of the primary functions of GSTs appears to be coping with environmental stress.

The initial classification of GSTs is based on their cellular location. In insects, GSTs exist as microsomal and cytosolic proteins. As microsomal GSTs have not yet been found to play a role in insecticide resistance (Enayati *et al*., 2005), they will not be discussed any further. Cytosolic GSTs were initially classified according to their elution profile, but this system was replaced when immunologically distinct classes were identified and named Class I and Class II (Fournier *et al*., 1992). Class I genes appear to be encoded by multigene families in insects, while Class II appears to be encoded by a single gene (Enayati *et al*., 2005).

As sequence data mounted, it became clear that the Class I/Class II naming systems were inadequate, with newly discovered isozymes not fitting into either class. A new naming system orthologous to the mammalian system of nomenclature was adopted. This system named the classes after letters of the Greek alphabet (Enayati *et al*., 2005). Using this system, based on amino acid sequence identity, it was found that Class I GSTs were unique to insects, and were split into the delta and epsilon GST classes (Ranson *et al*., 2000b). Class II GSTs were found to be orthologous to sigma GSTs, found in a range of organisms (Agianian *et al*., 2003). Insect encoded GSTs were also found in the diverse omega, theta and zeta classes (1,2 and 1 putative transcripts found in *An. gambiae*). Finally, there are 3 cytosolic genes, GSTU1-3, which cannot be assigned to any of these classes due to their low sequence identity to other insect GSTs (Ding *et al*., 2003). These GSTs' also constitute 3 of the 9 GSTs which do not exist in the cluster of 31 genes found in *An. gambiae* (Ranson *et al*., 2002).

GSTs are involved in the metabolism of numerous insecticides. The best known of these reactions is the induction of DDT resistance by a dehydrochlorination reaction (Clark and Shamaan, 1984). Their activity can, however, be induced by the insecticides fenpropathrin, propoxur, endosulfan, parathion, methyl paraoxon and carbaryl. Fumigants such as methyl bromide, acrylonitrile and ethylene oxide can also induce GST activity (Yu, 1996), highlighting their broad substrate specificity. GST metabolic activity is due to the water solubilisation of lipid products of GSH conjugation. Furthermore, by neutralising the electrophilic sites of the lipid substrates, they protect cellular components from electrophilic attack (Enayati *et al*., 2005). The mechanisms of GST-mediated pyrethroid resistance and protection against oxidative stress will be dealt with more comprehensively in chapter 4.

As with esterases and cytochrome P450s, elevated GST activity is achieved by gene amplification and amino acid substitutions (Ortelli *et al*., 2003, Ranson *et al*., 2002). A mechanism not commonly found in the other two superfamilies, but is a hallmark of GSTs, is the increase in diversity induced by alternative splicing. The NH_2-terminus of GSTs is highly conserved as it contains domains essential for the binding and activation of glutathione. The COOH-terminus contains residues involved in substrate specificity. Therefore, splicing a common NH_2-terminus onto various COOH-terminals can efficiently expand the diversity of

the enzyme substrate (Ranson *et al*., 2002). An example of this is the delta class AgGSTD1 gene which can produce four peptides with distinct and differing catalytic properties from a single transcript (Ranson *et al*., 1998). Similarly, a single sigma class GST gene in *An. gambiae* can produce 2 transcripts with a common 5' region but differing in the 3' region (Ding *et al*., 2003). It is therefore surprising that the role of alternative splicing in mosquitoes, particularly in anophelines, is not particularly well studied. Besides alternative splicing, transcriptional activation as well as mRNA stability has been implicated in GST upregulation (Yu, 1996).

The interplay between enzymes of the detoxification enzyme superfamilies is an important but highly under-examined aspect of vector biology. Yet, before this can be fully appreciated, it is necessary to complete the general schema of insecticide resistance outlined in figure 1.3.

All factors that contribute towards the decrease of the effective dose of the insecticide have been summarised – altered behaviour, decreased penetration and increased metabolism. Factors that render the dose ineffective are the final component of this discussion. These factors are target site resistance.

1.8 Negating the dose of insecticide resistance: Target site mutations

Adulticidal mosquito interventions all involve a nerve target. Therefore, alteration of the target site in such manner that the insecticide can no longer interact with the target and result in the inhibition of the relevant action (Ffrench-Constant *et al*., 1998). With the profusion of other mechanisms inducing resistance discussed before, why are target site mechanisms not more common, as this mechanism should be a simple method of producing a resistance phenotype? The answer is simply that an alteration of these usually structural proteins may come at a fitness cost, and may explain why so few target site mutations exist (Ffrench-Constant *et al*., 1998). Bearing this in mind, in the discussion of these mutations, careful consideration of the fitness costs of the relevant mutations will be made. Before the target site mutations can be discussed, however, a brief introduction to nerve function is necessary.

1.8.1 The synapse, the action potential and the potentiation of neural signals

The transmission of electrical impulses forms the basis of the nervous system. In electrically excitable tissues such as neurons or muscle, impulses are transmitted by action potentials; the change in electrical potential along the membrane of the cell (Barnett and Larkman, 2007). In the resting state, the membrane of electrically active tissues causes the membrane to depolarize, and the membrane potential can reach as high as +100 millivolts. This rapid increase of membrane potential past the threshold levels of the resting potential is known as the action potential. After the action potential has passed, the membrane rapidly re-polarises, dropping to a lower (more negative) potential than the initial resting state before returning to the resting state potential (Barnett and Larkman, 2007).

The change is mediated by the activity of two voltage gated channels, the sodium channel and the potassium channels. In the resting state, both channels are closed. During depolarisation, the potassium channel remains closed, while sodium channel opens and pumps sodium ions into the cell, making the membrane potential more positive. Repolarisation is initiated when sodium channels begin becoming inactive, while potassium channels become active. Repolarisation is mediated by the activity of potassium channels while sodium channels are inactive, resulting in an efflux of positive ions bring the membrane potential back to negative (Hodgkin and Huxley, 1952).

Action potentials have to pass along empty spaces where the electrically active cells are not physically in contact, such as the synaptic cleft between neurons. To allow the action potential to continue, neurotransmitters are released from the terminus of one axon to start the depolarisation of the next cell. One of these neurotransmitters is Gamma-amino butyric acid or GABA. GABA is presumed to be the most important inhibitory neurotransmitter in the nervous system. Therefore, the presence of GABA, as opposed to an excitatory neurotransmitter, such as glutamate, will result in a halting of the action potential, rather than its' potentiation (Kuffler and Edwards, 1958). GABA binds to GABA receptors found on neuromuscular junctions. GABA receptors, in contrast to sodium and potassium channels, are

therefore ligand-gated receptors rather than voltage-gated receptors (Krnjevic and Schwartz, 1967).

Acetylcholine (described in section 1.8.2), an ester of acetic acid and choline, is another important neurotransmitter. In contrast to GABA (described in section 1.8.3),, it is an excitatory neurotransmitter, which activates muscles. It mediates this by binding to nicotinic acetylcholine receptors, which results in the opening of the voltage gated sodium channels and an influx of sodium described in section 1.8.4), (Colquhoun *et al*., 1987). Acetylcholine is metabolised to inactive metabolites by the enzyme acetylcholinesterase. Acetylcholinesterase is abundant in synaptic clefts, and is required to clear excess acetylcholine from the system and is essential for proper muscle function. The central nervous system of insects is particularly rich in acetylcholine and acetylcholinesterase, with the nerve cord of cockroaches containing up to 15x the amount of acetylcholinesterase of mammals (Wigglesworth, 1953).

It is therefore clear that acetylcholinesterase, GABA receptors and sodium channels are essential for proper nervous function. Therefore, targeting these enzymes/receptors are effective methods of pest control. Unfortunately, structural modifications in these proteins have lead to widespread resistance. Furthermore, as one protein is the target for more than one insecticide class, target site mutations often confers cross resistance to unrelated insecticides (Brogdon and McAllister, 2004).

1.8.2 Altered acetylcholinesterase activity

Acetylcholinesterase is essential for the termination of neural impulses. Therefore, organophosphates (OPs) and carbamates which target this enzyme will result in continued neural firing due to the accumulation of acetylcholine. These insecticides carbamylate or phosphorylate the active site serine moiety (Corbett, 1974). As such, a structural alteration/replacement of the serine residue would render the acetylcholinesterase insensitive (Weill *et al*., 2004).

Typically, alterations in mosquito acetylcholinesterase (AChE) results in high carbamate and low OP resistance (Hemingway *et al*., 2004). In *Cx. pipiens* an amino acid substitution of

G119S was found. The glycine residue lies at the base of the active site gorge, and results in steric hindrance during substrate binding due to the substitution with the bulkier, more polar serine (Weill *et al*., 2004). The G119S mutation in the *Ace-1* gene has been found in *Cx. pipiens pipiens*, *Cx. pipiens quinequefasciatus* and *An. gambiae* and has been found to confer high levels of resistance (Hemingway *et al*., 2004). Altered AChE has been demonstrated to be the most common mechanism of OP/carbamate resistance in mosquitoes central America (Hemingway *et al*., 2004).

As acetylcholinesterase is a crucial enzyme for neural function, it is possible that mutations in this protein may have a fitness cost. This would appear to be true, as fitness costs associated with altered AChE has been reported in vitro, with mutations that affected its' catalytically efficiency reducing the stability of the enzyme (Shi *et al*., 2004). In laboratory strains of *An. gambiae* homozygous for the G119S mutation, it was found that these mosquitoes were more likely to die during pupation (Djogbenou *et al*., 2010). This finding was mirrored in field populations of *Cx. pipiens*, where mosquitoes with AChE mediated resistance had a longer development time and resulted in smaller adults (Bourguet *et al*., 2004). It has been postulated that the fitness cost incurred by these resistant individuals are not based on resource-based trade offs, but rather due to hyperactivation of the nervous system due to the altered AChE activity (Rivero *et al*., 2011).

1.8.3 The GABA receptor and the *rdl* mutation

The GABA receptor consists of five subunits arranged around an ion channel. When the GABA molecule binds to the receptor, it results in a flow of chloride ions which results in hyperpolarisation, the period after the action potential when the membrane potential is even more negative than during the resting phase, and consequently inactive (Macdonald *et al*., 1989). This receptor is the target of cyclodienes and phenyl pyrazoles which would prevent the gating of the channel, negating the inhibitory effect of GABA, thus resulting in neural hyperexcitation (Bloomquist, 1993, Narahashi *et al*., 1992). The discovery of the alanine 302 serine mutation in natural populations of *D. melanogaster* resistant to dieldrin prompted the

naming of this gene the resistance to dieldrin (*rdl*) gene (Ffrench-Constant *et al*., 1998). In rare cases, the alanine is replaced with a glycine (Zhang *et al*., 1994).

The alanine302 mutation lies within the transmembrane region of the *rdl* subunit which is presumed to be the primary constituent of the ion channel lining (Hemingway *et al.*, 2004). This mutation is both interesting and unique within the array of target site mutations, as it serves a dual purpose in conferring resistance. It not only occupies the insecticide binding site, causing steric hindrance, but also destabilises the desensitized state preferred by xenobiotics (Zhang *et al*., 1994). As there are no secondary mutations conferring GABA receptor mediated resistance, it is assumed that the ala302 mutation was the most evolutionarily effective, as well as least costly in terms of fitness (Ffrench-Constant *et al*., 1998). This hypothesis is supported by the fact that this mutation persists in *Drosophila* long after the selection pressure has been removed. This suggests a lack of a fitness cost (Hosie *et al*., 1997). The only reported fitness cost in *rdl* homozygotes appears to be a slight increase in temperature sensitivity (Remnant *et al*., 2013).

1.8.4 The sodium channel and the *kdr* mutation

The sodium channel is of particular significance to this study, as it is a major resistance mechanism in one of the strains used in this study. The significance of this will be expanded upon in chapter 2.

Voltage gated sodium channels (hereafter referred to as sodium channels) are vital to the propagation of action potentials. These channels are large multimeric proteins, consisting of an α-subunit and one or more β-subunits. The α-subunit contains the ion-conducting pore, as well as the other elements essential to the functioning of the channel. Without the α-subunit the channel cannot open, looses ion selectivity and cannot be rapidly in activated. These properties, however, cannot occur without the β-subunit, as these components modify the kinetics of the gating of the channel (Catterall, 2000).

The α-subunit consists of four similar domains, each of which contains six α helical transmembrane segments (Catterall, 2000). Structurally, the sodium channel differs from the

potassium channel by the presence of a highly conserved intracellular loop connecting domains III and IV. This loop serves as a hinged lid that that binds to the intracellular pore of the channel to inactivate it (Kellenberger *et al*., 1996, Yu and Catterall, 2003).

Knockdown resistance (*kdr*) was first observed in the housefly *Musca domestica* (Busvine, 1951). It was determined that mutations in the sodium channel rendered the insect less susceptible to the neurotoxic effects of DDT and pyrethrins, but no other classes of insecticides. This resistance could not be negated by the use of synergists. The housefly was also the source of the discovery of a second resistance trait, called super *kdr*, conferring even greater resistance to DDT and certain pyrethroids (Soderlund and Knipple, 2003).

In *An. gambiae*, the *kdr* mutation was initially discovered as the L1014F mutation (Martinez-Torres *et al*., 1998). Later, a second *kdr* mutation, L1014S was discovered (Ranson *et al*., 2000a). As these mutations were discovered in west and east Africa respectively, L1014F is referred to as West African *kdr* and L1014S as east African *kdr*. These geographical boundaries are not strict and co-occurrence of both mutations have been reported in *An. gambiae* from Gabon (Pinto *et al*., 2006) as well as *An. gambiae* from the Democratic Republic of the Congo (Choi *et al*., 2010).

Unlike the previously described target site mutations, numerous substitutions can produce a *kdr* phenotype. The L1014F phenotype has been reported in *An. gambiae*, *Cx. pipiens*, *Leptinotarsa decemlineate* and *M. persicae*. Alternative primary resistance-inducing mutations have been described in *Heliothis virescens* (V410M) and *Boophilus microplus* (F1538I). Enhancing mutations have been described in *Blatella germanica* and many other *kdr* polymorphisms associated with resistance have not been fully characterised yet (Soderlund and Knipple, 2003).

The multitude of *kdr*-type mutations should be an indication that this mechanism of resistance is not as simple as the previously described target site mutations. A clue to this is in the names given by the IRAC classification to the insecticides which target this channel. Carbamates and OPs are AChE inhibitors, cyclodienes are GABA receptor antagonists, while DDT and pyrethroids are sodium channel modulators (IRAC, 2012). The complexity of this system is

highlighted by the fact that the *kdr* mutation is not equally effective at inhibiting all sodium channel modulators. *Kdr* resistance is most strongly associated with DDT resistance, most weakly associated with Type II pyrethroids (deltamethrin) and has an intermediate effect of Type I pyrethroids such as permethrin (Reimer *et al*., 2008).

The biology of the L1014F mutation provides a working hypothesis about why this could be. The *kdr* mutation is most strongly associated with DDT resistance, then permethrin resistance, and finally deltamethrin resistance. This is also the order of increasing efficacy of these insecticides on the sodium channel, with DDT exerting the weakest effect, permethrin an intermediate effect and deltamethrin having the strongest effect (O'Reilly *et al*., 2006). The L1014F mutation shifts the channel to a more positive potential, promoting closed state inactivation. In L1014F mutants up to 70-80% of the sodium channels never open. DDT and pyrethroids preferentially bind to open state channels, and the opening of the channel is particularly important for the binding of Type II pyrethroids (Davies *et al*., 2007). Therefore, the L1014F mutation does not prevent binding of the insecticide to its' target site as with the *Ace-1* and *rdl* mutations, but rather reduces the bioavailability of binding sites (O'Reilly *et al*., 2006). The mutation therefore slows down the penetration of the insecticide into the electrically active target cells. As Type II pyrethroids are the most active agents against channels, they would require the lowest concentration of insecticide to exert an effect (Davies *et al*., 2007). This may underlie the differing association of *kdr* with the three different insecticides.

The assessment of fitness costs due to the *kdr* mutation have been difficult to determine. *Kdr* in association with amplified carboxylesterases was demonstrated to result in a reduced response to alarm pheromones in *M. persicae* (Foster *et al*., 1999). Houseflies homozygous for *kdr* alone exhibited behavioural differences related to temperature (Foster *et al*., 2003). It has also been demonstrated that field caught *An. gambiae* homozygous for *kdr* were more susceptible to oocyst maturation after an infectious blood meal (Alout *et al*., 2014). It has also been suggested that where *kdr* and *Ace-1* mutations co-exist the *kdr* mutation compensates for the fitness cost associated with *Ace-1* in the absence of insecticide (Berticat *et al*., 2008). The fitness cost associated with *kdr*-mediated resistance is far less researched than fitness costs

associated with *Ace-1* mutations. Whether this is due to a lack of research or reflects a lack of fitness cost is debatable. Acetylcholinesterases, like many other esterases, are well researched in *Culex*. Although gene amplification is a mechanism commonly associated with metabolic resistance, it has been found that this mechanism is also prevalent in target site mutations. This alleviates the negative effects associated with the reduced neural function associated with target site mutations. Gene amplification in target site mutations results in "permanent heterozygotes" that cope better than target site homozygotes (Bass *et al*., 2013).

1.9 Integration of metabolic detoxification systems: a 3 phase system of biotransformation in insects

Elucidation of the mechanism underlying insecticide resistance is an important tool. Synergist studies could be used to implicate enzyme systems in resistance. Biochemical analysis of enzyme activity is also a useful tool to understand the metabolic mechanisms underlying resistance (Penilla *et al*., 1998). Screening for target site mutations is also a useful way of not only determining the mechanism of resistance, but also predicting cross resistance. The rapid improvement of screening technologies is a testament to this fact, with the rapid identification of *kdr*, *rdl* and *Ace-1* mutations (Bass *et al*., 2007, Bass *et al*., 2010, Essandoh *et al*., 2013). Recently, the first report of a PCR assay for a mutation conferring metabolic resistance in *An. funestus* has been reported (Riveron *et al*., 2013). This negates the need for laborious biochemical assays, and regardless of whether any more mutations of this nature are discovered, this represents great progress in the resistance screening for vector control interventions.This is, however, only a first step and DNA-based diagnostic assays for metabolic resistance is still not commonplace.

The aim of these diagnostic tools is to rapidly determine the mechanisms of resistance within populations. This risk of this approach, however, is that it makes it too tempting to look for simple solutions for resistance mechanisms that may be multifactorial. By simply searching for elevated enzyme activity, or a list of overexpressed genes, it is easy to forget that different enzyme systems may interact with each other, or that metabolic resistance may alter the efficacy of target site mutations (Mitchell *et al*., 2014, Zhong *et al*., 2013).

In mammalian systems, many of the enzyme systems that are involved in biotransformation of toxicants are homologous to those in xenobiotic detoxification. In the mammalian system, it is well established that biotransformation occurs in three-phase system (Rose and Hodgkinson, 2004). Although the concept of three-phase detoxification has been described in insects (Yu, 2008), they are not as well known or examined in insects, and the tendency is to focus on phase I metabolism (M.Paine, personal communication.). In this study, the nature of phasic detoxification system will be examined. A summary of the generalised scheme of three-phase detoxification is given if figure 1.3

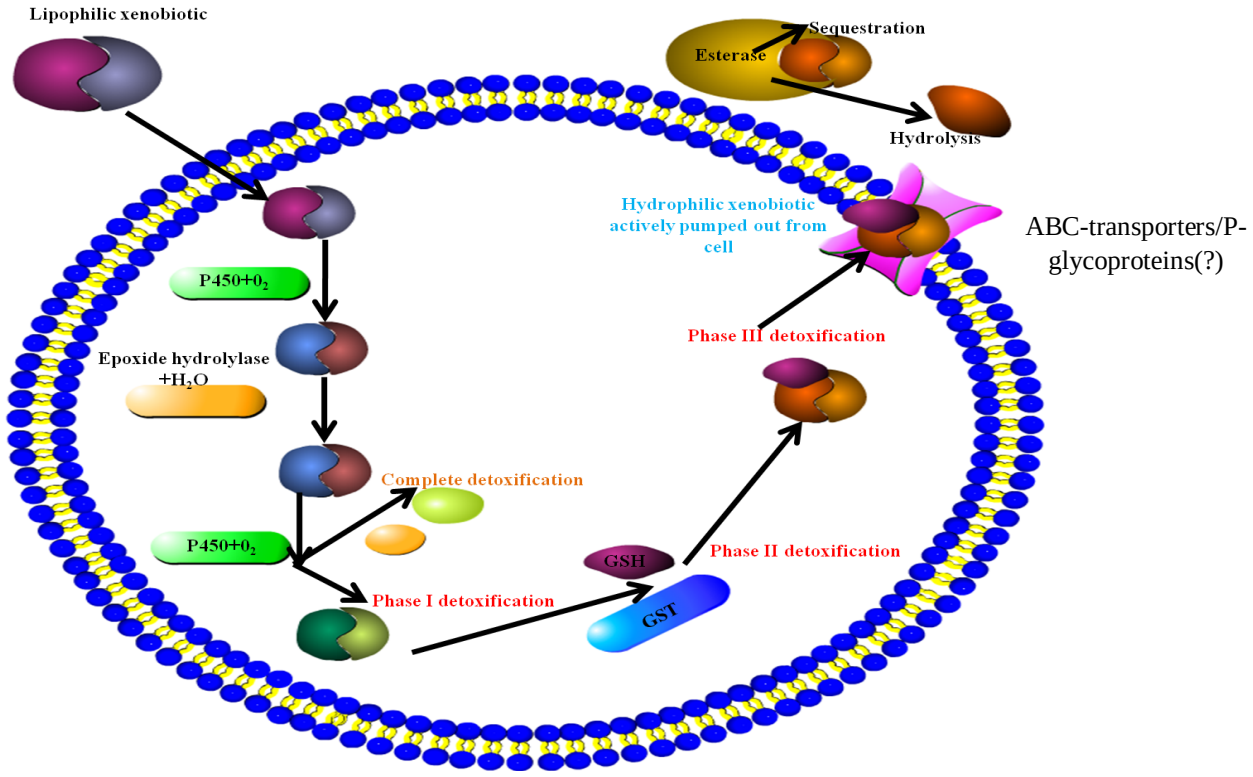


Figure 1.3: The three phase biotransformation system resulting in the detoxification of xenobiotic compounds. Lipophilic xenobiotics passively diffuse into the cell, where they are subjected to modification by Cytochrome P450s and other mononoxygenases/hydrolases. This constitutes Phase I of the biotransformation process. This can either result in complete detoxification or preparation for the conjugation reaction that constitutes Phase II reactions. The most common Phase II reaction is the GST-mediated conjugation of glutathione. The now water-soluble xenobiotic is removed by active transport (Phase III). Esterases are capable of sequestration and hydrolysis of xenobiotics and therefore do not fit into one specific stage of biotransformation. (Based on Rose and Hodgkinson, 2004, Sheehan *et al*., 2001, Yu, 2008).

1.9.1 Phase I detoxification

Phase I reactions are a group of reactions which typically introduce polar groups into a molecule, thus preparing the molecule for later potential conjugation reactions. The primary aims of Phase I reactions is to begin the process of making lipophilic xenobiotics more water soluble. These reactions are typically oxidation, hydrolysis or reduction reactions, although reduction reactions do not result in the molecule that is ready to proceed to the next stage of biotransformation (Rose and Hodgkinson, 2004). The key enzymes in this phase are esterases and cytochrome P450s.

The cytochrome P450 system is the Phase I enzyme of primary importance in insects, as well as all other eukaryotes due to their broad substrate specificity. These enzymes can catalyse epoxidation, hydroxylation, *o*-dealkylation, sulfoxidation, desulfuration and dehalogenation reactions, as well as various reduction reactions. These reactions are the most crucial for pesticide metabolism (reviewed in Yu, 2008). Epoxidation, sulfoxidation and desulfuration results in activation of insecticides, while demethylation and hydroxylation results in detoxification (Yu, 2008).

Phosphatases and carboxylesterases are the most important Phase I hydrolases in pesticide metabolism and are referred to as A (not inhibited by OPs) and B (inhibited by OPs) esterases according to the system proposed by Aldridge (Aldridge, 1953a,b). Epoxide hydrolases and Flavin containing monoxygenases are important non P450 Phase I enzymes in mammals and although they exist in insects, their role, if any, in insecticide resistance has not been elucidated (Yu, 2008).

1.9.2 Phase II reactions

Phase I reactions result in the addition of functional groups such as epoxide, hydroxyl and carboxyl groups. The groups can undergo further conjugations with endogenous molecules such as sugars, amino acids, phosphate and glutathione. This results in a compound that is less toxic and more hydrophilic, and as such, more readily excreted. Phase II reactions are therefore all conjugation reactions (Rose and Hodgkinson, 2004).

Three conjugation reactions exist in insects. Type I reactions require an activated conjugating agent to combine with the substrate to form a conjugated product. Type II reactions involve the activation of the substrate. The substrate then becomes an activated donor which conjugates with an endogenous molecule. In Type III reactions conjugation is direct, and requires no activation steps. Type I and II conjugations therefore require high energy intermediates to proceed. These types of conjugations involve glucose, sulphate, and phosphates for Type I reactions and amino acids for Type II conjugations. Type III conjugations involve the conjugations of halogens, epoxides and glutathione among others (Rose and Hodgkinson, 2004). Glucose conjugation is associated with the metabolism of allethrin, DDT, carbofuran, propoxur and carbaryl. Sulphate conjugation is involved in the metabolism of phenolic compounds. The most important conjugation reaction, though, is that of glutathione (Yu, 2008).

Glutathione conjugation is mediated by GSTs. Although GSTs have a specific requirement for reduced glutathione (GSH), they have broad substrate specificity with a low energy conjugation reaction. The reaction catalysed by GSTs results in the formation of mercapturic acids. These products are highly water soluble (Habig *et al*., 1974). To be removed from the lipophilic environment of the cell, these products need to be removed by active transport, and are pumped out of the cell. Once outside, the now lipophilic compound will not passively diffuse back into the cell (Habig *et al*., 1974).

In mammalian systems, Phase III detoxification consists of the active transport of now more hydrophilic molecule (Rose and Hodgkinson, 2004). Although increased efflux pumps have long been known to be a common drug resistance mechanism in parasites (Rai *et al*., 2013), no analogous system was believed to exist in insects. Recently, however, candidates have emerged that may represent a Phase II detoxification system in insects. Members of the ATP binding cassette (ABC) transporters are a ubiquitous family of membrane proteins. Members of this family, particularly P-glycoproteins, are ATP-dependent efflux pumps, capable of actively removing xenobiotics from the cell (Buss and Callaghan, 2008).

ABC transporters have been indirectly implicated in insecticide resistance in a number of ways. Inhibition of their activity resulted in significantly increased mortality to a variety of

insecticides including cypermethrin, endosulfan and methyl parathion (Buss and Callaghan, 2008). Increased expression of ABC transporters have been reported in *Ae. aegypti* (Bariami *et al*., 2012) and *An. arabiensis* (Jones *et al*., 2012b). Although it is generally agreed that the exact mechanism whereby ABC transporters mediate resistance is not fully elucidated, largely due to a lack of functional data, they are the best candidates for Phase III transporters in insects (Dermauw and Van Leeuwen, 2013).

Although Phase I enzymes such as esterases and cytochrome P450s are capable of metabolising insecticides directly, many of their products are fed into later phases of detoxification. It is therefore important to consider the interactions of the components of the metabolic detoxification system, rather than examining them in isolation.

1.10 Environmental stress

An understanding of the biology of malaria vectors is crucial to cope with the ever-increasing threat of insecticide resistance. This fact has not gone unnoticed, as it is clear from the abundance of research into the subject, from macroscopic issues such as behaviour or physiology to the molecular basis of these phenotypes. There is debate about the use of field material versus laboratory material (Huho *et al*., 2007, Norris *et al*., 2001). Arguments can be made for the merits of both, but the reality is that regardless of what type of material is involved, most experiments take place in a laboratory. A laboratory consists of ideal conditions, the purpose of which is to simplify a study by removing extraneous variables. The major hallmark of a study conducted in a laboratory, therefore, is the lack of environmental stress.

There have been previous studies to examine the effect of environmental stress on the biology of mosquitoes. These have included studies on inter and intraspecific larval competition. Less work has been done on the effect of environmental stress on the insecticide resistance phenotype, although this is not completely unexamined (Halliday and Feyereisen, 1987, Hodjati and Curtis, 1999a, Hodjati and Curtis, 1999b, Kulma *et al*., 2013, Swain *et al*., 2008). A key question that needs to be examined is the role of the stress response as a whole on the insecticide resistance phenotype.

There is much cross-talk between the different metabolic systems of the organism (Hull, 2012, Korsloot *et al.*, 2004). Each of these are affected by various environmental stresses, which in turn affects the activity of systems they interact with (Korsloot *et al*., 2004). The resistance mechanism in mosquitoes, particularly in *Anopheles*, is often examined in isolation, or without consideration of how it affects other biological processes, with the obvious exception of fitness costs. The effect of various environmental stressors on the response to insecticides, as well as how these responses interact are factors that need to be investigated.

Another question that needs to be asked is how the insecticide responses of laboratory-reared mosquitoes are altered when exposed to a stress that they would encounter in the natural environment. Studies on laboratory reared mosquitoes are often criticised for not being representative of what happens in the natural environment. As laboratory reared mosquitoes are often a necessary tool of vector biology, the results of this study would therefore contribute to making laboratory-based studies more applicable to the natural situation. Before the rationale of this experiment can be expanded upon, it is necessary to understand a little about the cellular response to environmental stress.

1.10.1 Environmental stress in insects

A definition of environmental stress is "a condition evoked in an organism by one or more environmental factors that bring an organism near to or over the edges of its' ecological niches" (Korsloot *et al.*, 2004). Stress is also a transient state which involves a syndrome of physiological responses, which are subsequently accompanied by the processes to counteract the consequences of the stress (Korsloot *et al.*, 2004).

Depending on the severity of the stress, the danger of this scenario is that cellular homeostasis can be disturbed. It is therefore crucial that mechanisms are in place to cope with the disturbance. Figure 1.5 illustrates several groups of stressors, as well as the responses they include.

The physical stressors (lack of food, heat) and metabolic stressors resulting in oxidative stress, aspects which in this context can be considered "environmental factor(s) causing a change in a

biological system which is physically injurious" (Hoffmann and Parsons, 1991) were examined in this study. Stress responses have both an ecological and evolutionary role (Sorensen and Loeschcke, 2007) and yet their involvement in vector biology remains largely unexamined. Details of previously performed studies will be provided in the introductions of the relevant chapters regarding a particular stress.

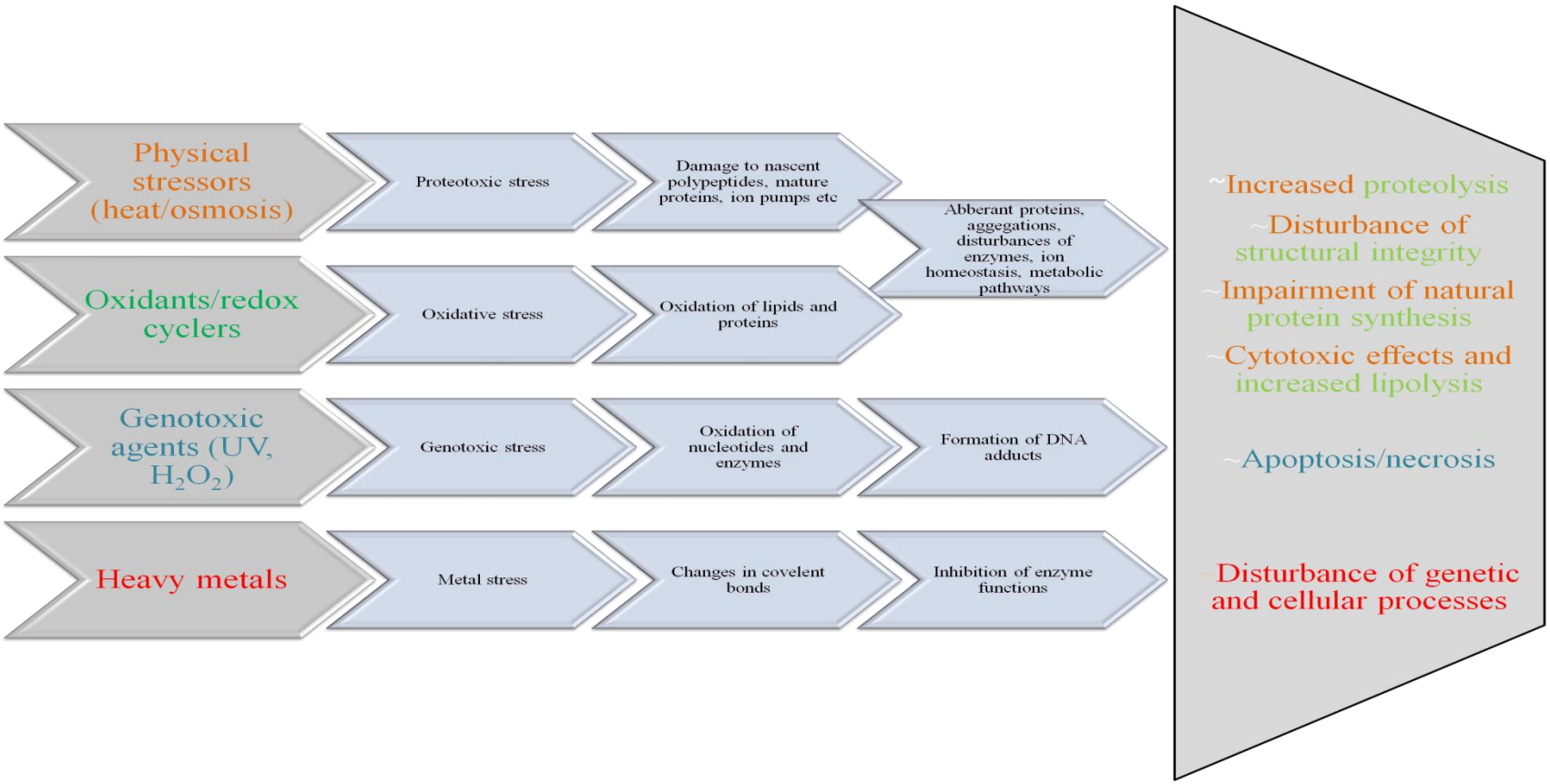


Figure 1.4: **Environmental stressors and their cellular outcomes**. The four important classes of environmental stressors commonly encountered by invertebrates are physical stressors such as heat or osmotic stress, oxidative stressors, genotoxic agents and heavy metals. Each results in a specific cascade of macromolecule-specific damage. The cellular outcomes of each stress is highlighted in the purple block, with the yellow and green text representing typical damage by physical and oxidative stressors, blue text representing the results of genotoxic stress and orange representing the results of metal stress (adapted from Korsloot *et al.*,2004) .

1.11 Context and Rationale

Malaria has played an important role in the history of South Africa. The disease took many lives, including the entire city of Ohrigstad. The Lesetedi Valley in Tzaneen was known as "death valley" due to the many malaria-related deaths (Hansford, 1984). The city of Mashishing was originally known as Lydenburgh (place of suffering) due to the malaria burden. Yet 110 years later, South Africa stands on the threshold of eliminating (zero local transmission) malaria (Maharaj *et al*., 2012).

Operational research on insecticide resistance is crucial for South Africa to reach its malaria elimination goals (Maharaj *et al*., 2012). Basic biological research is also crucial. Yet, unlike other countries in Africa, the biological material, particularly vector species, are not easy to come by. Therefore, for practical and logistical reasons, laboratory reared mosquitoes remain an important starting point for basic vector biological research.

The behavioural plasticity of *An. arabiensis* is a particular concern for vector control efforts. The second concern is their apparent climactic hardiness. The capacity of *An. arabiensis* to tolerate hotter, drier conditions was reported in 1979 (Coluzzi *et al*., 1979). Under unstressed conditions, when *An. gambiae* and *An. arabiensis* larvae occur together, *An. gambiae* will outcompete its siblings. Under stressed conditions, *An. arabiensis* tend to outcompete *An. gambiae* (Kirby and Lindsay, 2009, Paaijmans *et al*., 2009). As such, *An. arabiensis* makes an excellent candidate species to examine the effect of environmental stress in anophelines.

Anopheles arabiensis and *An. funestus* therefore represent a particular challenge for vector biologists. This is particularly significant in South Africa as the country embarks on a malaria elimination programme, with the aim of zero local transmission by 2018 (Blumberg *et al*., 2014, Moonasar *et al*., 2013). As vector control is the mainstay of effective public health programmes aimed at the elimination of vector borne diseases (Hill *et al*., 2005), control of these two vectors is of crucial importance. Yet these two DVS remain at a research disadvantage in comparison to *An. gambiae*. Therefore, a better understanding of the biology of these vectors is paramount. The broad aim of this study was to understand the mechanisms

that improve or affect vector competence. One of the most important of these factors is vector fitness. In this study the major life history parameters that will be examined are development time and longevity.

The primary aim of this study was to assess the role of environmental stress on the insecticide resistant phenotype and longevity of laboratory reared strains. *Anopheles arabiensis* and *An. funestus* have been chosen as the species to be studied as they are the primary vectors of malaria in Southern Africa (Sinka *et al*., 2010). The nature of these stressors examined are summarised in figure 1.5.

The primary objectives of the study were:

1. To determine the effect of larval nutritional stress on the insecticide resistant phenotype of adults.
2. To determine if cellular stress responses affect the insecticide resistance phenotype and longevity of the adult.
    a. Does defence against oxidative stress affect the insecticide resistance phenotype?
3. To determine whether common physical stressors affect the insecticide resistant phenotype/longevity of adults.
    a. What is the role/effect of multiple blood-meals on the insecticide resistant phenotype/longevity?
    b. What effect does thermal stress have on the insecticide resistant phenotype/longevity?
4. To assess whether interplay between metabolic and target-site resistance exists.
5. To consolidate the interplay and crosstalk between the stress response systems and the metabolic detoxification system.

1.12 Common Methods

A number of methods were common to most of the chapters in the study. These methods will be detailed here, and this section will be referred to in later chapters.

1.12.1 Mosquito rearing

All mosquitoes were housed in the Botha de Meillon insectary. Standard insectary conditions are described in Hunt *et al*. (2005). Mosquitoes were housed at 25^{o}C (±2^{o}C) and at 80% (±5%) relative humidity. Unless otherwise stated, mosquito larvae to be used in experiments were fed four times daily on a diet of powdered dog biscuits and brewer's yeast. For routine purposes, larval food is not quantified, but rather fed to the discretion of the colony caretaker. To minimise variation, mosquitoes that would be used in the experiments would be reared by the experimenter. For purposes of colony maintenance, adults were offered a non-human (guinea pig) blood meal twice weekly and allowed to oviposit weekly. For the purposes of blood feedin experiments, a single, healthy adult volunteer on no medication served as a bloodmeal. The ethical clearance waiver approving blood meals are provided in appendix 2.

During the course of the study several terms were used which may require defining. A cohort is defined as a group of adults from a same-generation egg batch. As such, when different cohorts are used, they are drawn from different egg batches. The use of different cohorts constitutes biological replicates. The biological replicates are typically divided into a number of replicates or groups subjected to the experimental treatments. These multiple repeats of experiments within biological replicates are referred to as technical replicates.

1.11.2 WHO and synergist bioassays

WHO bioassays were performed as described by the World Health Organisation (WHO, 2013). In brief, unless otherwise stated, 20-25 three to five day old unmated, non-blood fed adults, always separated by sex, were gently introduced into WHO bioassay holding tubes lined with paper. Standard discriminating doses of these insecticides are given in Tables 2.2.1 and Table 2.2.2. They were allowed to acclimatise to the conditions for 1 hour before being introduced into a bioassay tube lined with paper impregnated with an insecticide to simulate an insecticide-treated surface. The adults were exposed to the insecticide for 1 hour before the adults were re-introduced to the holding tube for recovery. After exposure the adults were allowed *ad libitum* access to a 10% sucrose solution and mortality was scored 24 hours post

exposure. Adults were moved between the adjoining holding tube and exposure tube by gently blowing them from one chamber to the next.

For every WHO bioassay, two controls were used. The first control constituted a handling control. These mosquitoes would be exposed only to paper treated with a carrier solvent. This ensured that the physical treatment of the mosquitoes did not lead to elevated mortality. The second control was an environmental control. This involved sample of adults exposed to the holding tube only, to ensure that no environmental factor contributed to mortality. If mortality in both controls was between 3 and 10%, Abbot's formula ([% test mortaliy-% control mortality]/[100-%control mortality]x100) was applied to correct for mortality. If control mortality exceeded 10% the results were discarded.

Synergist bioassays that were performed varied from the standard CDC synergist protocol. Details of this methodology are provided in appendix 4 (protocol for the preparation of synergists). In these assays mosquitoes were exposed to synergist impregnated paper for an hour prior to a standard WHO insecticide exposure. Three standard synergists were used. To synergise cytochrome P450s 4% butoxide PBO was used. This concentration was used as previously published (Brooke *et al*., 2001). The concentrations for the synergist triphenyl phosphate (TPP) and diethyl maleate (DEM), used to synergise esterases and GSTs respectively, were optimised at 20%. The optimal concentration was determined as the highest possible concentration that did not result in mortality when adults were exposed to paper treated with the concentration in question for 1 hour. For synergist bioassays insecticide-induced mortalities of synergist-treated individuals were compared to those of adults exposed to insecticide only. Synergist bioassays not only had the environmental and handling controls described for the standard WHO bioassays, but included a control that required a group of adults to be exposed to the synergist only for an hour. This ensured that the synergist alone did not induce mortality. Details of the preparation of synergist papers are detailed in the appendices.

1.11.3 Biochemical Assays

Quantification of detoxification enzyme activity is a common technique in many of the following chapters.

1.11.3.1. Protein Quantification

As specific activity rather than relative product quantification is used, the amount of protein in each sample is quantified using the Bradford assay. The principle of this assay, originally described in 1976 (Bradford, 1976), is applied to a microwell plate assay. Unless otherwise stated, samples for all biochemical analysis were prepared as follows: 3-5-day old non-blood fed adults were cold terminated and stored at -70^{o}C. Samples to be used were never stored for longer than 6 weeks to maintain enzyme activity. Individual mosquitoes were homogenised in Milli-Q water without protease inhibitors using a Qiagen tissue lyser II (Whitehead Scientific/Thermo Scientific). Protein was quantified using Bradford reagent (phosphoric acid/Coomassie brilliant blue/water; Bio-Rad) with Bovine Serum Albumen (Sigma Aldrich) used to calibrate a standard curve. The absorbance at 595nm was determined using a Spectramax 190 96-well plate reader (Separations-Original supplier). For all other spectrophotemetric assays, unless otherwise stated, a Multiskan Ascent 96 well plate spectrophotometer (Thermo Scientific) was used to determine optical density.

1.11.3.2 The cytochrome P450 assay

Haeme peroxidase activity is used as a measure of cytochrome P450 activity (Brogdon *et al.*, 1997). To 20µl of crude homogenate a mixture containing a final concentration of, 1.38 x 10^{-8} M 3,3'5,5' tetramethyl benzidine (Elmans' reagent)(Sigma) dissolved in a solution consisting of 15ml methanol, 15ml sodium acetate pH 5.0, was added. The reaction was allowed to proceed in 80µl of 0.0625M potassium phosphate pH 7.2. The reaction was stabilised by adding 3% hydrogen peroxide to a final concentration of 0.7M. This reaction was allowed to proceed for 2 hours at room temperature and the product produced was quantified by an end

point reading of 650nm. The product was quantified by using a cytochrome c (Sigma Aldrich: C-3006) standard curve and results are reported as cytochrome c equivalents/mg/ml. Assays were only accepted if the accompanying standard curve had an r^2 value exceeding 0.95. Cytochrome C served as a positive control and homogenate free samples served as blank/negative values.

1.11.3.3 The general esterase assay

A final concentration of 22 mM of either α or β-napthyl acetate substrate in a volume of 120µl of sodium phosphate buffer pH 7.2 was added to 20µl of crude insect homogenate. The reaction was allowed to proceed on ice for 30 minutes before being terminated by a staining solution containing a final concentration of $7.18x10^{-7}$M of Fast blue RR salt (Sigma) dissolved in a solution of 0.1M sodium phosphate buffer pH7.0 supplemented with 5% sodium dodecyl sulphate (Sigma) and diluted with 2.25ml distilled water. The reaction was allowed to stain for 5 minutes at room temperature before the product was quantified by an end point read at 570nm. The product was quantified using an α- and β-napthol standard curve with results reported as α/β napthol equivalents/mg/ml (Penilla *et al.*, 1998). Assays were only accepted if the accompanying standard curve had an r^2 value exceeding 0.95. Alpha and Beta napthol served as a positive control and homogenate free samples served as blank/negative values.

1.11.3.4 Glutathione S-transferase activity

GST activity was determined using the model substrate 1-chloro-2,4-dinitrobenzene (CDNB)(Sigma). To 10µl of crude homogenate a working solution of 4.92×10^{-8} M reduced glutathione and $1.56x10^{-8}$M CDNB dissolved in 1ml methanol in a final volume 200µl in 0.25M sodium phosphate pH 6.5 and the rate of CDNB conjugation was monitored kinetically at 340nm for 5 minutes (Brogdon and Barber, 1990). GST activity was quantified as the rate of conjugation over 5 minutes. Assays were calculated only only values were enzyme conjugation was found to be in the linear range. The enzyme activity was considered successful if the amount of product formed was more than the uncatalysed reaction of the blank wells included in the plate. The formula used for calculating GST activity in Appendix 5, formula 1.

1.11.4 Determination of *kdr* genotype

Various assays exist to determine *kdr* genotypes including polymerase chain reaction (PCR) assays and restriction fragment length polymorphism (RFLP) assays. In this study, a hydrolysis probe (Taqman™) assay was used to determine *kdr* genotype for both the L1014F and L1014S mutations (Bass *et al*., 2007, Bass *et al*., 2010). In brief , the assays for L1014F and L1014S requires two standard primers, used at a working concentration of 10µM: kdr-Forward: CATTTTTCTTGGCCACTGTAGTGAT and kdr-Reverse: CGATCTTGGTCCATGTTAATTTGCA. To discriminate between the two polymorphisms the following Taqman MGB probes are used for L1014F and L1014S detection respectively: WT: VIC-CTTACGACTAAATTTC and KdrW: 6FAM-ACGACAAAATTTC; WT: VIC-CTTACGACTAAATTTC and KdrE: 6FAM-ACGACTGAATTTC. A 20µl reaction contains 1-2 µl of genomic DNA, 1X SensiMix DNA kit (Quantace) , 800nM kdr-Forward primer, 800 nM kdr-Reverse, 200 nM of probe WT, 200 nM of probe KdrW OR KdrE and sterile water to 20ul (e.g. 5 ul). The reaction was cycled for 10 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds. No template controls were used, as well as specimens that were confirmed for their *kdr* status by sequencing were used as positive and negative controls.

1.11.5 Oxidative stress assays

1.11.5.1 Quantification of oxidative stress by protein carbonyl content

Protein carbonyl content was used as a measure of oxidative stress damage (Reznick and Packer, 1994, Vontas *et al*., 2001). Unless otherwise stated, 0.15g of 3-5 day old, unfed mosquitoes, separated by sex were homogenised in 50mM sodium phosphate buffer pH 7.0 with the inhibitors dithiotreitol (DTT), phenylmethyl sulfonyl fluoride (PMSF), aproptinin, and leupeptin were added to a final concentration of 0.5µg/ml. The buffer was supplemented with Tween-20 to a final concentration of 0.1%. Samples were then centrifuged at 10 000g for 3 minutes at 4^{o}C. The supernatant was collected and used as the protein for the ditrophenyl hydrazine (DNPH) derivitization reaction.

For each protein preparation, the sample was split into two equal portions, one to be derivitivized and another non-derivativized sample to serve as a baseline control. For derivitivized samples 1:1 volumes of 10mM DNPH in 2mM hydrochloric acid were added to the protein preparation. To the control non-derivitivized samples 1:1 volumes of 2mM hydrochrloric acid were added to the sample. From this point, derivitivized and non-dervitivized samples were treated in the same manner.

After the addition of DNPH, samples were vortexed to ensure even distribution and incubated in the dark at 25^{o}C for 60 minutes. Samples were vortexed every 15 minutes. After this incubation samples were precipitated with 10% trichloroacetic acid (TCA) (2:1 acid: sample) and incubated on ice for 10 minutes. Samples were then centrifuged for 15 minutes at 10 000g at 4^{o}C. The supernatant was then discarded and the pellet resuspended in 1ml ethanol: ethyl acetate solution (1:1).The procedure was repeated twice (a total of three ethanol: ethyl acetate washes). The pellet was finally suspended in a half volume as 6M guanidine hydrochloride dissolved in 10mM sodium phosphate buffer pH 2.3. The optical density at 366nm was determined using a Spectramax 190 (Thermo Scientific). The formula for calculating carbonyl content is detailed in the appendices. Although the actual extinction co-efficient for DNPH at 366nm is 0.022$\mu M^{-1}.cm^{-1}$, this value was adjusted to 0.011$\mu M^{-1}.cm^{-1}$ to account for the pathlength of the 96 well plate. The formala for calculating carbonyl content in found in Appendix 1, formula 2.

1.11.5.2 Quantification of catalase activity

This method was optimised from a 1972 spectrophotometric method to calculate catalase activity by hydrogen peroxide (H_2O_2) consumption (Sinha, 1972). In this experiment, single specimens were homogenised in 100mM Potassium phosphate buffer pH 7.0 without any protease inhibitors. Fifty µl of mosquito homogenate was used as starting material and to this Potassium phosphate buffer was added to a final concentration of 1.4M. 0.23M H_2O_2 was added to this solution, as was a 200µl Potassium dichromate acetic acid solution (1:1). This reaction was monitored kinetically at 570nm for 30 minutes. Catalase activity was determined

by calculating H_2O_2 consumption, determined by a H_2O_2 standard curve, and the formula for calculating catalase activity is detailed in appendix 5, forumala 3.

1.11.5.3 Quantification of glutathione peroxidase activity

This method was optimised from the procedure used to determine glutathione peroxidase activity in the brown leaf hopper (Vontas *et al.*, 2001). In this procedure, samples were prepared for assay in the same manner as the catalase assay. A total of 160µl of the working solution was added and incubated at room temperature. The working solution consisted of 1mM EDTA (Ethylenediamine tetracetic acid) pH 8.0, 1mM reduced glutathione (GSH) solution, 1unit/ml GSH reductase, 0.2mM NADPH in 50mM Sodium phosphate buffer pH 7.0. This reaction was monitored kinetically for 15 minutes at 340nm. The formula for determining peroxidase activity is given in the appendices. Again, the actual extinction co-efficient of NADPH is 0.00622 $\mu M^{-1}.cm^{-1}$, but the co-efficient used was adjusted for the pathlength of a 96 well plate (0.000373 $\mu M^{-1}.cm^{-1}$). The formala for calculating carbonyl content in found in Appendix 1, formula 4. .

1.11.6 Statistical Methods

For all statistical analysis the Statistix 8 programme (Talahassee, Florida, USA) and Statistica 10 (Statsoft, Oklahoma, USA) were used. To compare the mean of a treatment to that of a control, a 2-sample t-test with a confidence interval of 95% was used. When multiple means were compared, a 1 way Analysis of variance (ANOVA) with a confidence interval of 95% was used. The Tukey HSD test was used as a post-hoc test for ANOVA. Longevity was determined using the Kaplan-Meier estimator, with the Log-Rank test used as a measure of significance. All figures were prepared either in MS Excel or Statistix 8 and compiled in MS Powerpoint.

Chapter 2: Baseline characterization of the *Anopheles arabiensis* strain SENN DDT and the *Anopheles funestus* strains FUMOZ and FUMOZ R

2.1.1 Introduction

As detailed in chapter 1, *Anopheles funestus* and *An. arabiensis* are the major malaria vectors found in Southern Africa. Laboratory strains of these two species form the basis of all the experiments that will be described in this study. The use of laboratory strains is often critiqued for a variety of reasons. The most often cited critique is that due to optimal feeding and reduced flying requirements, that laboratory reared mosquitoes have high sugar stores. This in turn would bias studies involving energetic activities such as flying, and activities that would affect vector competence such as host seeking and biting (Day and Van Handel, 1986).

Reproductive function in laboratory-reared mosquitoes is also found to be affected. The longer *An. gambiae* males remain in colony, the shorter the length of their sperm, but the larger their testes become (Baeshen *et al*., 2014). It is, however, not easy to predict which type of mosquito, laboratory-reared or free-living, will perform better by given parameter, as laboratory-reared mosquitoes do not consistently out- or underperform their wild counterparts (Huho *et al*., 2007). For example, it has been suggested that wild males have better lipid reserves and as such would perform better reproductively and survive better than laboratory-reared mosquitoes (Huho *et al*., 2007). In a comparison of the fitness of laboratory-reared *An. arabiensis* for release in a Sterile Insect Technique programme in South Africa, it was found that no relative fitness costs were incurred in the laboratory-reared strain to be used by the programme (Munhenga *et al*., 2011).

Laboratory-reared mosquitoes are a powerful tool for examining genetic involvement in phenotypic characteristics by eliminating extraneous variables. Although the risk exists that laboratory-reared mosquitoes are not representative of wild populations, different characteristics are affected variably by colonisation. Reproductive characteristics have already been mentioned, but metabolically demanding characteristics may be biased and amplified in

laboratory strains. Immunity, which has a high metabolic demand, is one of those characteristics. Insecticide resistance has been shown to enhance immunity in laboratory strains of *Culex pipiens* (Vézilier *et al*., 2012). Similarly, insecticide resistant *Cx. pipiens* have been shown to produce more prophenoloxidase and therefore have better immunity than their susceptible counterparts (Cornet *et al*., 2013). In both cases, the same response is absent in wild mosquitoes.

In contrast, age of colonisation has not been shown to affect thermal limits in both *An. arabiensis* and *An. funestus* (Lyons *et al*., 2012). It has been suggested that the longer a strain remains in colony, the longer lived they will be (Rajatileka *et al*., 2011). This has been disproven by a study of insecticide susceptible strains of *An. arabiensis* kept in colony for varying lengths of time that did not differ significantly in longevity (Essop, 2014). Therefore, although it should be treated with caution, and can always be confirmed in wild populations, laboratory-reared strains are a valuable resource and starting point in vector studies.

The broad aims of this study were therefore twofold. The first aim was to determine the effect of various environmental stresses on the expression of insecticide resistance and life history of laboratory strains. The ideal conditions of the insectary do simplify studies, but what happens when environmental stress is applied?

The second aim of this study was to investigate some of the biological differences between insecticide resistant and susceptible anopheline mosquitoes. The fitness cost of insecticide resistance in *Culex* mosquitoes is a well-studied subject (Hardstone *et al*., 2010, Rivero *et al*., 2011). Surprisingly, not much work has been done on anophelines, with one study on laboratory *An. funestus* (Okoye *et al*., 2007) being the most cited example. This study also aimed to examine whether insecticide resistant and susceptible strains only differed in their capacity to metabolise xenobiotics, or whether they also displayed broader biological differences. To this extent, a review of the strains to be used during the course of this study is given.

2.1.2 *Anopheles arabiensis* from Sudan and the SENN strains

Anopheles arabiensis is the major malaria vector in Sudan, with *An. gambiae* s.s. making up a very small portion of the population, occurring only in the humid, southernmost areas (Petrarca *et al*., 2000). Resistance to DDT, pyrethroids and malathion are common (Abdalla *et al*., 2008, Abdalla *et al*., 2014, Himeidan *et al*., 2011) but resistance to bendiocarb still appears to be absent (Abdalla *et al*., 2008). Several interesting discoveries have arisen from this particular *An. arabiensis* population, including the fact that *An. arabiensis* is preferentially attracted to pregnant women (Himeidan *et al*., 2004). The Sudanese *An. arabiensis* population was also the first anopheline population in Africa where malathion resistance was reported. The resistance mechanism was particularly interesting as it was synergised by the esterase inhibitor TPP and yet no increase in the activity of general α and β-esterase activity was detected (Hemingway, 1983). This type of malathion resistance was later demonstrated to be mediated by a malathion-specific detoxification enzyme called malathion carboxylesterase (Karunaratne and Hemingway, 2001).

In 1980, an *An. arabiensis* population from Sennar was colonised, and named SENN. In 1995, a sub-population of this strain was selected on 4% DDT to produce the SENN DDT strain. The SENN DDT strain, and initially the Sudanese *An. arabiensis* populations from Gezira and Sennar, posed an interesting problem. Studies of the laboratory strain and field populations found no correlation between the *kdr* mutation and DDT and permethrin-induced mortality (Abdalla *et al*., 2008, Matambo *et al*., 2007). This posed the question of whether *kdr* alone could produce a resistance phenotype (Brooke, 2008). This question will form a major theme in this study. Subsequent field studies have since shown a positive association between the *kdr* mutation and DDT/pyrethroid resistance, as well as evidence of metabolic resistance in the Sudanese population (Himeidan *et al*., 2011). The same does not fully hold true for the laboratory strain SENN DDT, where it will be demonstrated in chapter 3 that L1014F RR homozygotes also can succumb to DDT intoxication.

As SENN DDT is the primary resistant strain that will be examined in this study, a good understanding of the basis of the resistance mechanisms is crucial. A full bioassay and

biochemical screening of the strain was done on the F16 generation in 2007, but it is likely that the situation has changed since then. This chapter will therefore provide the latest bioassay data on this strain. Initial biochemistry data showed that only GST activity was upregulated in both males and females, and esterase activity was significantly elevated only in males of the strain (Matambo *et al*., 2007). Microarray analysis of the strain demonstrated that only a single gene CYP9L1 was upregulated (Nardini *et al*., 2012). Both these results are inconsistent with the multiple insecticide resistant phenotype of this strain, and required further investigation.

2.1.3 Laboratory strains of *Anopheles funestus*

Southern Mozambique is a fertile area for research into *An. funestus* and the importance of this population was highlighted in chapter 1. It has been demonstrated that this population, like many other *An. funestus* populations, is endophilic. Although the *An. arabiensis* that occurs in sympatry with this population has a higher human landing index, the *An. funestus* population has a higher sporozoite infection rate. Furthermore, while the *An. arabiensis* population fluctuates throughout the year, the *An. funestus* population remains stable (Kampango *et al*., 2010; Mendis *et al*., 2000). It has been shown in this population of *An. funestus* appears to favour cooler temperatures, resulting in larger females (Charlwood and Bragança, 2012; Charlwood *et al*., 2003). Females in this population that engage in host seeking rapidly after a blood meal show significantly higher oocyst loads (Charlwood and Tomás, 2011).

Perhaps the most significant factor about the *An. funestus* population of Southern Mozambique is that they were the first *An. funestus* population to be colonised. The FUMOZ strain was established from this population and the FUMOZ R strain was selected from this strain in 2005 (Hunt *et al*., 2005). The insecticide susceptible strain FANG was colonised from Angola in 2000. *Anopheles funestus* research has often lagged behind that of *An. gambiae*, with the difficulty in colonising this species playing a particular role (Coetzee and Fontenille, 2004). This highlights the importance of laboratory strains. The FANG strain is still a fully susceptible *An. funestus* strain and is the only available susceptible reference strain available

for insecticide resistance studies in *An. funestus*, and is thus commonly used as a susceptible control (Morgan *et al*., 2010).

An example of the usefulness of colony material was demonstrated in the establishment of a *Plasmodium berghei* infection model in *An. funestus*. In April 2013 it was demonstrated that a wild *An. funestus* could maintain a *P. berghei* infection system (Xu *et al*., 2013). In June of the same year, it was demonstrated that not only could *An. funestus* support a *P. berghei* infection system, but also that parasite load was inversely correlated with pyrethroid resistance capacity (Lo and Coetzee, 2013). Yet perhaps the most important example of the importance of laboratory strains in insecticide resistance research, particularly in *An. funestus*, is the elucidation of the mechanism of pyrethroid resistance in the *An. funestus* population of Southern Mozambique.

2.1.4 CYP6P9 and pyrethroid resistance in *Anopheles funestus*

In 2001, after the South African malaria epidemic of 2000, it was demonstrated by biochemical and synergist studies that cytochrome P450 mediated resistance was the most likely underlying mechanism of the pyrethroid resistance in the population (Brooke *et al*., 2001). After the FUMOZ strain was established, studies of the insecticide resistance mechanism accelerated rapidly. In September of 2005, the establishment of the FUMOZ R strain (selected from FUMOZ using permethrin) confirmed cytochrome P450 mediated pyrethroid resistance in the strain. An increase in GST activity without accompanying DDT resistance was also demonstrated (Hunt *et al*., 2005). In December of the same year twelve partial gene sequences for cytochrome P450s of the CYP4, 6 and 9 families were isolated (Amenya *et al*., 2005). Also in December of that year an integrated genetic and physical map, based on studies of the colonised material, was published (Wondji *et al*., 2005). In 2008, the overexpression of a single P450 gene, CYP6P9, was confirmed in both egg and adult stages (Amenya *et al*., 2008). Shortly afterwards, it was found, again in colony material, that CYP6P9 was 25X overexpressed and CYP6P4 was 51X overexpressed in resistant females. It was suggested that gene duplication was responsible for this gene's contribution to resistance (Wondji *et al*., 2009). In 2010, the full sequence of CYP6P9 was isolated from both resistant

and susceptible laboratory *An. funestus* strains. Interestingly, the genes from the two different strains shared only 99.3% sequence identity, with the differences lying outside the conserved region of the gene. In the same study, a duplicated CYP6P9 gene, called CYP6P13, was also isolated (Matambo *et al*., 2010). Since then two tandemly duplicated genes have been identified in populations from Malawi and Mozambique, although these are referred to as CYP6P9a and CYP6P9b (Riveron *et al*., 2013).

Although it has been demonstrated that pyrethroid resistance does not incur a fitness cost in *An. funestus* (Okoye *et al*., 2007), in this study, the most recent bioassay and biochemical data for the resistant *An. funestus* strains is presented.

In this chapter, a baseline assessment of the insecticide resistance phenotypes and the mechanisms of resistance of the strains that were used in this study was performed. The insecticide resistance phenotype, synergist profile and metabolic detoxification enzyme activity was assessed.

2.2 Materials and Methods

2.2.1 Materials

The following strains were analysed in this study:

Anopheles arabiensis

- SENN: A strain colonised from Sennar, Sudan in 1980.

- SENN DDT: A strain selected from SENN by exposure to 4% DDT. This strain has been under continuous selection since 1995, and is still presently selected on 4% DDT. This strain displays resistance a number of insecticides.

Anopheles funestus

- FANG: A strain colonised from Calueque, Angola in 2002. It is fully susceptible to all insecticides.

- FUMOZ: A strain colonised from Mozambique in 2000. It displays pyrethroid and carbamate resistance.

- FUMOZ R: A strain selected for pyrethroid resistance from the FUMOZ strain, originally by exposure to 1.5% permethrin. The strain has not been selected in over 10 years, but still maintains pyrethroid and carbamate resistance.

The *An. arabiensis* strain SENN DDT was analysed for insecticide resistance phenotype profile, synergist profile and metabolic detoxification enzyme activity. The *An. funestus* strains FANG (susceptible), FUMOZ (resistant, unselected) and FUMOZ R (resistant, selected) strains were used in this experiment. The insecticide resistance profile and metabolic detoxification enzyme activity of the FUMOZ and FUMOZ R strains were determined with the FANG strain serving as a baseline control. The rearing conditions of these strains are described in chapter 1.

2.2.2 Baseline resistance screening of SENN DDT

The baseline resistance of SENN DDT was determined by standard WHO bioassay. The details of this procedure are described in chapter 1. WHO bioassays were performed using 4% DDT, 0.75% permethrin, 0.05% deltamethrin and 5% malathion. The total numbers of individuals used in the assays are presented in table 2.2.1

Table 2.2.1: Sample sizes for standard and synergist exposures using *Anopheles arabiensis* SENN DDT**.**

Treatment	Exposure	Female	Male
Standard Exposures	Control	1210	1206
	DDT 4%	1259	1230
	Permethrin 0.75%	1815	1446
	Deltamethrin 0.05%	1425	1599
	Malathion 5%	1700	1747
	Dieldrin 4%	536	461
	Bendiocarb 0.1%	443	211
Synergist Exposures	DDT Control	1259	1230
	PBO+DDT	1264	1119
	TPP+DDT	1279	1100
	Permethrin Control	1851	1446
	PBO+Permethrin	1752	1404
	TPP+Permethrin	1212	1183
	Deltamethrin Control	1425	1559
	PBO+Deltamethrin	1287	1100
	TPP+Deltamethrin	1170	1163
	Malathion Control	1700	1747
	PBO+Malathion	1666	1604
	TPP+Malathion	1186	1183

2.2.3 Synergist Assays of SENN DDT

Synergist assays were performed on all four insecticides. 4% Piperonyl butoxide (PBO) was used as a cytochrome P450 synergist as described by Brooke *et al.* (2001). Range finding assays were performed to find the optimal concentrations for the remaining synergists. The highest concentration that did not induce death in synergist-only treated specimens, 20% TPP and 20% DEM, was used to synergise esterases and GSTs respectively. Controls were prepared as described for synergist exposures as described in chapter 1. Sample sizes are given in table 2.2.1

2.2.4 Baseline resistance screening of FUMOZ and FUMOZ R

The baseline resistance of the FUMOZ and FUMOZ R was determined by standard WHO bioassay. The details of this procedure are described in chapter 1. WHO bioassays were

performed using 0.75% permethrin, 0.05% deltamethrin, 0.05% lambda cyhalothrin 0.1% bendiocarb (table 2.2.2).

Table 2.2.2: Samples sizes for *An. funestus* standard exposures by colony.

	Treatment	Female	Male
FUMOZ Exposures	Unexposed Control	1125	1100
	Bendiocarb	1259	1230
	Deltamethrin	1815	1446
	Lambda-cyhalothrin	1425	1599
	Permethrin	1700	1747
FUMOZ R Exposures	Unexposed Control		
	Bendiocarb	1259	1230
	Deltamethrin	1815	1446
	Lambda cyhalothrin	1425	1599
	Permethrin	1700	1747

2.2.5 Biochemical screening of the *Anopheles funestus* strains

Cytochrome P450 activity (measured as haeme peroxidase activity), GST activity and general esterase activity was determined in FANG, FUMOZ and FUMOZ R as described in chapter 1. For each strain and sex, 96 individuals were assayed.

2.3 Results

2.3.1 Baseline resistance of SENN DDT

The SENN DDT strain showed resistance to DDT, permethrin, deltamethrin, malathion, dieldrin and bendiocarb. Although dieldrin and bendiocarb mortality was high, they were still resistant according to current WHO guidelines (WHO, 2013). Females were significantly more resistant than males to DDT (2-sample t-test: $p<0.01$, $t=6.61$), permethrin (2-sample t-test: $p<0.01$, $T=-3.5$) , deltamethrin (2-sample t-test: $p<0.01$, $T=-5.1$) and dieldrin (2-sample t-test: $p=0.04$, $t=2.51$) but not malathion (2-sample t-test: $p=0.06$, $t=-1.91$), bendiocarb (2-sample T-

test: p=0.78, t=-0.27). Results for general exposures are shown in figure 2.3.1 A (females) and 2.3.2 A (males). Results were analysed in terms of current WHO guidelines were 98%-100% mortality indicates insecticide susceptibility, while mortality of 98% and under indicates resistance (WHO, 2013).

2.3.2 Synergist assays of SENN DDT

DDT resistance was not successfully synergised in either males or females using any of the synergists. PBO caused significant synergism of permethrin in females (2-sample t-test: $p<0.01$, $t=4.36$) as well as males (2-sample t-test: $p<0.01$, $t=7.94$). PBO also caused significant synergism of deltamethrin, almost completely negating P450-mediated resistance in males (2-sample t-test: $p<0.01$, $t=-7.94$) and females (2-sample t-test: $p<0.01$, $t=-8.9$). Although TPP caused significant synergism of malathion in males (2-sample t-test: $p<0.01$, $t=7.54$) and females (2-sample t-test: $p<0.01$, $t=15.83$), the only other insecticide it had a significant effect on was deltamethrin where it resulted in a significant increase in mortality in males (2-sample t-test: $p=0.02$, $t=2.72$) and females (2-sample t-test: $p<0.01$, $t=3.38$). Synergist results are

summarised in figure 2. 3.3 B-E (females) and figure 2.3.4 B-E (males).

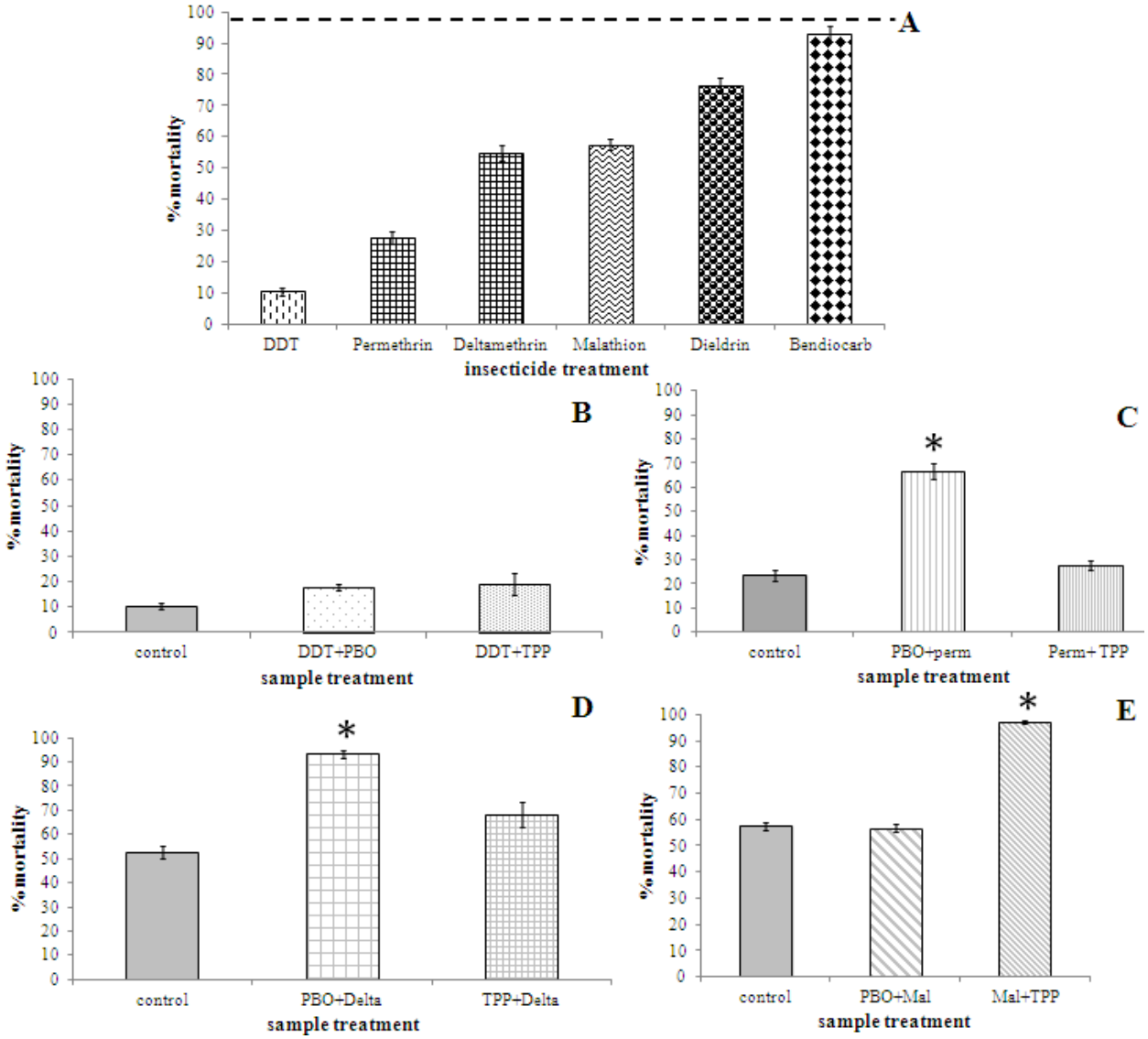


Figure 2.3.1: Standard and synergist exposures of SENN DDT *Anopheles arabiensis* females. Mean mortalities from baseline exposures (A) demonstrate that SENN DDT females were resistant to all insecticides assayed according to WHO criteria. The cut-off for susceptibility is indicated by the dashed line. As unexposed control mortality did not exceed 5% at any point, it is not indicated on the figure. Any significant increases in DDT-induced mortality (B) permethrin-induced mortality (C) deltamethrin resistance (D) and Malathion-induced mortalirt (E) is indicated by an asterisk (*)

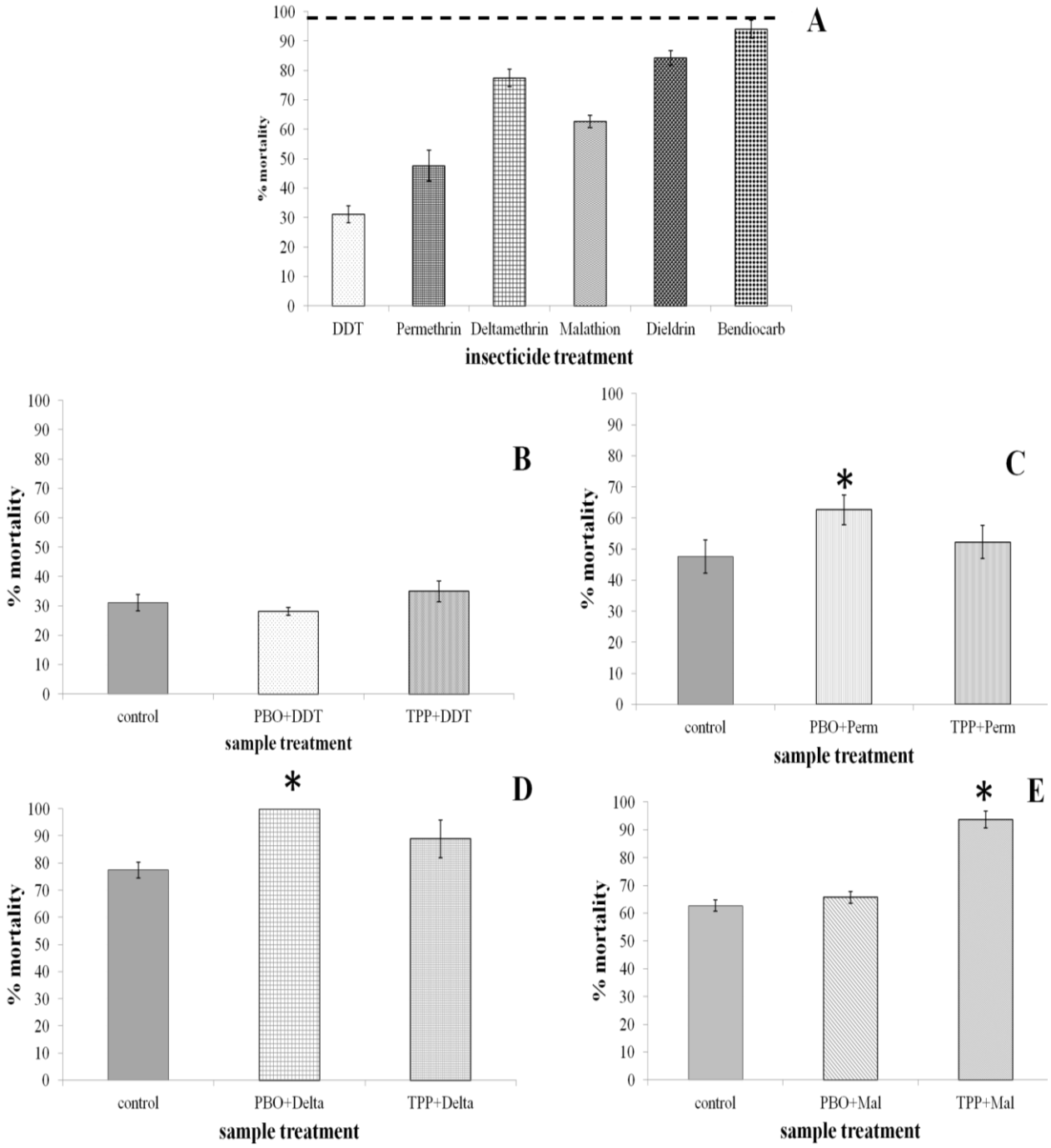


Figure 2.3.2: Standard and synergist exposures of SENN DDT *Anopheles arabiensis* males. Mean mortalities from baseline exposures (A) demonstrate that SENN DDT females were resistant to all insecticides assayed according to WHO criteria. The cut-off for susceptibility is indicated by the dashed line. As unexposed control mortality did not exceed 5% at any point, it is not indicated on the figure. Any significant increases in DDT-induced mortality (B) permethrin-induced mortality (C) deltamethrin resistance (D) and Malathion-induced mortalirt (E) is indicated by an asterisk (*)

2.3.3 Resistance screening of FUMOZ and FUMOZ R

As the FUMOZ and FUMOZ R strains were reported to be resistant to carbamates and pyrethroids, their susceptibility to bendiocarb, deltamethrin, lambda-cyhalothrin and permethrin was analysed. There was not a large difference in the resistance profiles of the FUMOZ and FUMOZ R strains. FUMOZ R was significantly more resistant to permethrin than corresponding FUMOZ samples for both males (2-sample t-test: $p<0.01$, $t=6.81$) and females (2-sample t-test: $p<0.01$, $t=-3.38$). The only other difference is a significant decrease in bendiocarb mortality in FUMOZ R females (2-sample t-test: $p<0.01$, $t=5.2$). These results are summarised in figure 2.3.3.

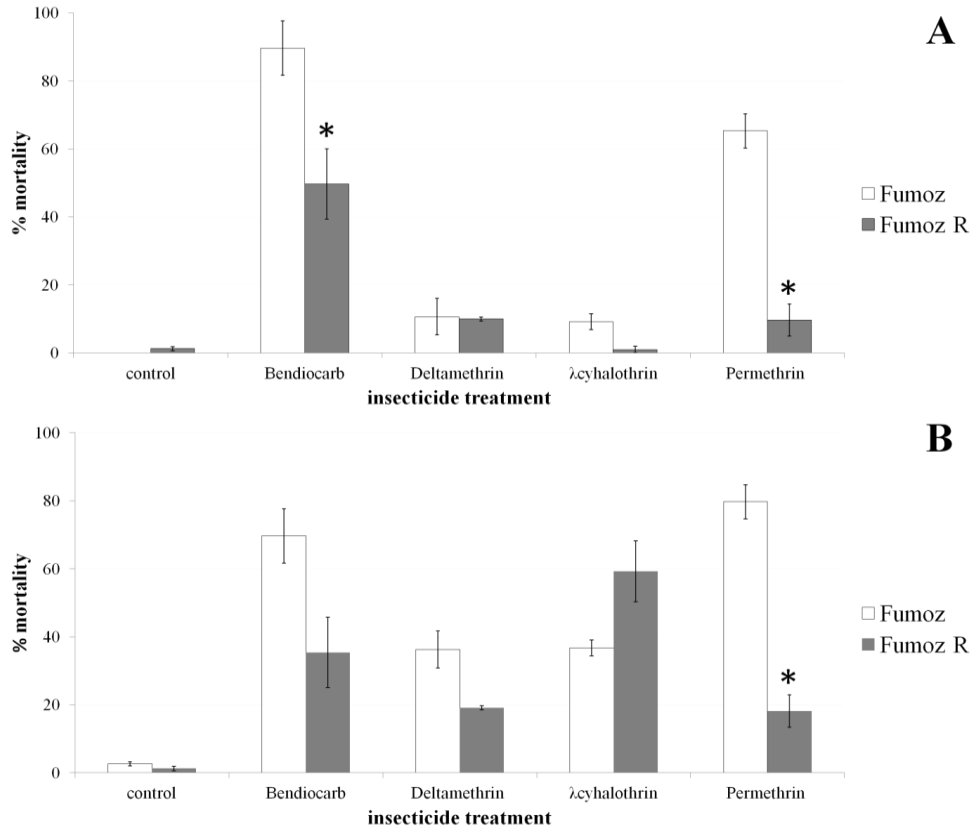


Figure 2.3.3: Baseline insecticide susceptibility levels of the FUMOZ and FUMOZ R *Anopheles funestus* strains. Permethrin resistance was significantly higher in FUMOZ R in both females (A) and males (B). There was no significant difference in mortality between FUMOZ and FUMOZ R for any of the other insecticides.Significant differences are indicated by an asterisk(*).

2.3.3 Biochemical analysis of the *Anopheles funestus* strains

The insecticide susceptible FANG strain consistently showed the lowest enzyme activity for all three classes of enzymes. In general, there was not much variation in enzyme activity in the FUMOZ and FUMOZ R strains. These results are summarised in figure 2.3.4. Cytochrome P450 (figure 2.3.4A) showed the greatest variation between the three strains. There was significant variation in the cytochrome P450 activities of FANG, FUMOZ and FUMOZ R males (1-way ANOVA: $p<0.01$, $F=9.61$) and females (1-way ANOVA: $p<0.01$, $F=11.9$). For females, FUMOZ R showed significantly higher P450 activity than FUMOZ (2-sample t-test: $p=0.02$, $t=-2.39$), as did male FUMOZ R (2-sample t-test: $p=0.04$, $t=2.06$). There was no significant difference in cytochrome P450 activity between males and females in FANG (2-sample t-test: $p=0.15$, $t=1.44$), FUMOZ (2-sample t-test: $p=0.51$, $t=0.67$) and FUMOZ R (2-sample t-test: $p=0.2$, $t=1.29$).

GST activity (figure 2.3.4B) of both FUMOZ and FUMOZ R was significantly higher than that of FANG males (1-way ANOVA: $p=0.01$, $F=1.83$) and females (2-sample t-test: $p<0.01$, $T=1.44$). There was a significant difference in the activity of FUMOZ and FUMOZ R females (2-sample t-test: $p<0.01$, $T=2.15$) but not males (2-sample t-test: $p=0.2$, $t=1.44$).

Alpha esterase activity of FANG females was significantly lower than FUMOZ and FUMOZ R females (1-way ANOVA: $p=0.02$, $F=4.25$). The same was true for males (1-way ANOVA: $p<0.01$, $F=10$). Alpha esterase activity was significantly higher in FUMOZ R males compared to FUMOZ males (2-sample t-test: $p=0.01$, $t=3.27$), This did not hold true for females (2-sample t-test: $p=0.3$, $t=1.05$) (figure 2.3.4C).

As with all the other enzymes, FANG males showed significantly lower β-esterase activity compared to both FUMOZ and FUMOZ R (1-way ANOVA: $p<0.01$, $F=15.3$) as did FANG females (1-way ANOVA: $p<0.01$, $F=7.52$). There was, however, no significant difference between FUMOZ and FUMOZ R females (2-sample t-test: $p=0.2$, $t=1.28$), but FUMOZ R males showed significantly higher activity than FUMOZ males (2-sample t-test: $p<0.01$, $t=2.83$) (figure 2.3.4D).

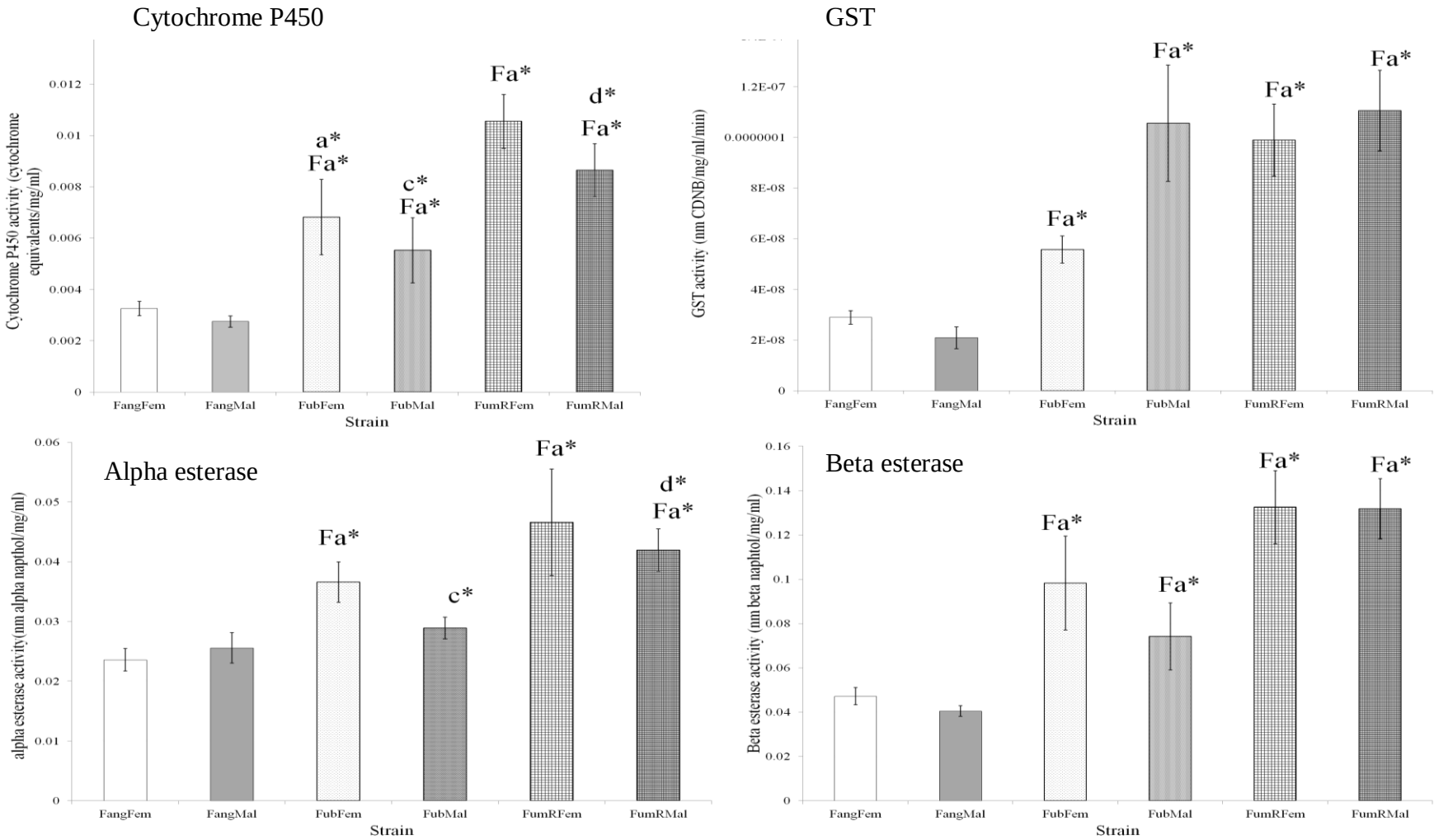


Figure 2.3.4: Detoxification enzyme activity in 3 laboratory strains of *Anopheles funestus*. The susceptible FANG strain (FANGFem: FANG females; FANGMal: FANG males) is compared to that of the unselected FUMOZ (FubFem: FUMOZ females; FubMal: FUMOZ males) and selected FUMOZ R strains (FumRFem: FUMOZ R females; FumRMal: FUMOZ R males). Asterisks marked Fa indicate a significant difference compared to the FANG strain of the same sex. Asterisks (*) with a letter indicate a significant difference between FUMOZ and FUMOZ R of the same gender.

2.4 Discussion

The resistance profile of the SENN DDT strain has changed significantly since 2007 (Matambo *et al*., 2007). In that study, the strain displayed high levels of resistance to all classes of insecticides. These includes 16.7% malathion mortality, 33.3% bendiocarb mortality, 12.1% DDT mortality, 24% deltamethrin mortality and 0% mortality for permethrin and dieldrin. This is no longer true, as some reistance phenotypes have subsequently waned. Resistance to dieldrin and bendiocarb has greatly reduced in both males and females (mortality for both insecticides in both sexes exceeds 90%). They are still, however, considered resistant according to WHO guidelines. The vast reduction in dieldrin resistance would initially suggest that the *rdl* mutation was lost. Since the 2007 study, continuous selection of the SENN DDT strain on 4% DDT continued until the F100 generation. After F100, generations were no longer monitored, but selection still continued at time of writing. The strain maintained its resistance to multiple insecticides despite being only exposed to DDT. The *kdr* mutation is fixed in this population (Matambo, 2007, Nardini *et al*., 2012), but DDT and permethrin-induced mortality still occurs, although at a low level. This suggests that DDT and permethrin resistance is primarily mediated by the *kdr* mutation. Synergist data appears to corroborate this data with the GST synergist DEM failing to synergise DDT resistance (data not shown). DDT resistance was significantly decreased by both PBO and TPP treatment, although the overall increase in mortality was not large. This suggests that *kdr* is the primary mechanism of DDT resistance in this strain. It is important to mention that DEM does not appear to be a good GST synergist in mosquitoes. As DEM is a glutathione scavenger (Sakurai *et al*., 2002) rather than a direct inhibitor of the enzyme, unlike inhibitors such as s-hexyl glutathione (Gopalakrishnan and Shaha, 1998), DEM synergist results should be treated with caution. Similar observations have been made previously (Ranson *et al*., 2001).

Although permethrin resistance can be mediated by esterases, there was no evidence for this mechanism in the SENN DDT strain. TPP synergism did not significantly increase permethrin-induced mortality. For both males and females, PBO significantly increased permethrin mortality without negating it completely. This suggests that both cytochrome P450s and *kdr* mediate permethrin resistance (Brooke and Koekemoer, 2010).

Deltamethrin is a type II pyrethroid and most weakly associated with the *kdr* mutation (Reimer *et al*., 2008). Resistance to type I and type II pyrethroids is often mediated differently, with type I pyrethroids being prone to esterase mediated resistance due to the ester bonds present in their structure (Young *et al*., 2005). These results are mirrored in SENN DDT where deltamethrin resistance, unlike permethrin resistance, appears to be almost completely mediated by cytochrome P450s, with very little involvement of the *kdr* mutation. This can be seen by the complete negation of the deltamethrinn phenotype by PBO synergism (figures 2.3.1. and 2.3.2).

In the F16 population, no increase in general esterase activity was observed in female SENN DDT, but malathion resistance was present. This was similar to the finding of Hemingway (1983). In the present day population, malathion resistance is synergised by the esterase inhibitor TPP. Unlike the Hemingway study, general esterase activity was found to be increased in this strain (detailed in chapter 3). Therefore, if malathion resistance was previously mediated by a malathion carboxylesterase, it is either no longer solely mediated in this manner, or malathion resistance is now completely mediated only by general esterases. This may explain the increased susceptibility to malathion.

It is curious that multiple resistances were observed both in 2005 and in the present study and yet previous attempts to characterise the resistance in this strain were not conclusive. According to the biochemical analysis of 2005, only GST activity was increased (Matambo *et al*., 2007), and according to the 2012 study, a single gene, CYP9L1 is upregulated (Nardini *et al*., 2012). These findings are incongruous with the phenotypic results. The biochemical data of 2005 may be explained by the fact that relative, rather than absolute specific activity was compared, where only specific activity was compared in this study.

The fact that only one overexpressed gene was found in SENN DDT may speak more about the genetic regulation of the resistance in the strain. Microarray analysis would only be able to pick up changes at the transcript level, and would not be able to detect altered activity due to post translational changes, particularly elevated enzyme activity due to post translational modification, differential trafficking or increased enzyme stability (Gygi *et al*., 1999). GSTs in particular are known to induce differing activity by using different splice variants (Ranson *et al*., 1998). This highlights the importance of unifying bioassay, transcriptional and enzymatic data.

Resistance to pyrethroids and carbamates is still present in both the FUMOZ and FUMOZ R strains. It is interesting as FUMOZ R has not been selected in many years, and the FUMOZ strain was never selected for resistance. The FUMOZ-Back Selection strain used in the *P. berghei* study was back-selected for permethrin susceptibility (Lo and Coetzee, 2013), but has subsequently regained pyrethroid resistance without selection (unpublished data) highlighting the fact that P450-mediated pyrethroid resistance does not incur a fitness cost in this strain (Okoye *et al*., 2007).

Pyrethroid and carbamate resistance levels did not differ greatly from the last survey of the laboratory strains (Stradi, 2011). Biochemical analysis was last performed in 2001, and as with the analysis of SENN DDT, the analysis was done on relative, rather than specific activity. Specific activity gives an absolute quantification of enzyme activity, whereas while using relative quantification of activity it is possible to overlook changes in enzyme activity due to relative rather than absolute quantification. Furthermore, in Brooke *et al.* (2001) the enzyme activity was compared to SKUQUA, a susceptible *An. quadriannulatus* strain as a baseline. In this study, the baseline comparison is a susceptible *An. funestus* strain.

It is of interest to note that CYP6P9 transcription is elevated in the FUMOZ R strain (Amenya *et al*., 2008, Christian *et al*., 2011), and it was found in this study that haeme peroxidase activity as a measure of P450 activity is increased, as is 7-ethoxycoumarin activity, a common measure of resistance-related enzyme activity (Wondji *et al*., 2014). Yet Western analysis showed no significant increase in CYP6P9 protein expression (Stradi, 2011). This suggests that the increased enzyme activity in FUMOZ R is not regulated only at the transcript level, but that enzyme activity in the resistant strain is altered so that it is more efficient. This has been corroborated by site directed mutagenesis studies that conferred the same level of pyrethroid metabolising capacity to CYP6P9 of the susceptible FANG strain as that of the resistant FUMOZ R strain (Wondji *et al*., 2014). This implies that in elevated P450 metabolic capacity is not only mediated by increased levels of P450 enzyme, but also due to the increased substrate specificity of the enzyme in the resistant strain.

When comparing the enzyme activity of the three laboratory strains of *An. funestus*, the most obvious result is that both FUMOZ and FUMOZ R have significantly higher levels of

cytochrome P450 activity than FANG, and that FUMOZ R males and females have significantly higher levels of activity than their FUMOZ counterparts. As this is the most diverse of the results, it reinforces the fact that cytochrome P450s are the primary mediators of pyrethroid resistance in FUMOZ R.

Similar to the results reported by Brooke *et al.* (2001), GST activity was found to be elevated in both FUMOZ and FUMOZ R in comparison to the susceptible FANG strain. There is, however, no significant difference in the GST activity between FUMOZ and FUMOZ R. As both the FUMOZ and FUMOZ R strains display pyrethroid resistance (deltamethrin, lambda cyhalothrin and permethrin), but only differ significantly in permethrin resistance, the initial permethrin selection is what drove the selection of the elevated P450 activity. The importance of the GST activity is not to be ignored though, and this will be expanded upon in Chapter 4. Both FUMOZ and FUMOZ R display resistance to bendiocarb, although not at significantly different levels. As both of these strains display increased α and β-esterase activity in comparison to FANG, this phenotype may be mediated by esterases. Of the two classes of esterases, FUMOZ R only has significantly higher α-esterase activity in comparison to FUMOZ. Beta-esterase activity does not differ significantly between FUMOZ and FUMOZ R.

In conclusion, SENN DDT mediates resistance to multiple types and classes of insecticides by multiple mechanisms. The *kdr* mutation appears to be the primary mediator of DDT and permethrin resistance in this strain. Pyrethroid resistance in *An. funestus* strains remains largely unchanged despite a lack of selection over many years. FUMOZ and FUMOZ R do not differ significantly in insecticide resistance with the exception of permethrin, where FUMOZ R has significantly lower mortality. This is likely a direct effect of the permethrin selection procedure. The pyrethroid resistance phenotype appears to be due to cytochrome P450 activity. The increased GST activity without DDT resistance in this strain remains significant.

Chapter 3: The effect of larval nutrition of the life history and expression of insecticide resistance in *Anopheles arabiensis*

3.1 Introduction

The larval environment is a crucial determinant of the fitness of emergent adult mosquitoes. Of the variety of challenges that anopheline larvae have to withstand, access to food is one of the most critical. One of the key differences between laboratory-reared and wild mosquitoes is that the former has access to optimal nutrition, and therefore fitness characteristics observed in the laboratory may not be reflective of the natural situation. The aim of this chapter was to investigate the effect of larval nutritional deprivation on the insecticide resistant phenotype in a laboratory strain of *Anopheles arabiensis*, as well as to examine whether a lack of nutrients affected larval development time in insecticide resistant and susceptible strains.

3.1.1 The biology of larval feeding

Mosquito larvae are usually classified into filter feeders, browsers (*Culex*) and predators (Toxorynchites). *Anopheles* mosquitoes engage in collecting and filtering, previously referred to as suspension or filter feeding. They consume ultrafine particulate matter, usually between the sizes of 50-0.5µm, from the organic surface covering the water, known as the surface microlayer (Clements, 2000). Mosquitoes do engage in active drinking of water, although they swallow their food bolus without taking in water. Water consumption rate usually increases with age (Clements, 2000).

Although micro-organisms are the primary dietary constituent of the aquatic stages of mosquito life, with biofilms being of particular importance, they also consume non-living organic matter. The detritus of digested plant material consumed by bacteria and fungi are an important constituent of this non-living component (Clements, 2000).

3.1.2 Larval nutritional requirements

3.1.2.1 Amino acids

Like all other insects and many vertebrates, *Aedes aegypti* larvae require 10 essential amino acids from their diets which they cannot synthesise naturally. Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine are essential for the development of this species (Goldberg and de Meillon, 1947). For *Culex pipiens*, the same 10 amino acids were crucial, but they also required asparagine (Singh, 1957). A lack of hydroxyproline and serine severely retarded growth in this species, and cystine was essential for pupation. Proline, which could be partially synthesised by *Culex pipiens*, was also essential for growth (Singh, 1957). Although data in *Anopheles* are not as well detailed, a complete diet that lacked amino acids resulted in larvae not surviving past the second instar (Goldberg and De Meillon, 1947).

3.1.2.2 Vitamins, Fatty acids and sterols

Mosquito larvae also require vitamins essential to life that cannot be synthesised. Without nicotinic acid (vitamin B3), panthothenic acid (vitamin B5), pyridoxine (vitamin B6), riboflavin (vitamin B2) and thiamine (vitamin B1), larvae did not survive past the second instar (Goldberg and de Meillon, 1947). Pupation did not occur without biotin, folic acid and the phospholipid compound choline (Clements, 2000).

All insects also have a strong dietary requirement for 20-carbon polyunsaturated fatty acids known as eicanasoids. They obtain 18-carbon polyunsaturated fatty acids from their diets, and many insects can synthesise 20-carbon fatty acids utilising desaturation and elongation pathways. Mosquitoes, however, are not capable of elongating 18-carbon fatty acids. Instead, they shorten longer fatty acids by retroconversion pathways. Fatty acid deficiencies have serious consequences for mosquitoes, resulting in reduced survival of immature stages. The most common symptom of fatty acid deficiency is the inability to fly (Clements, 2000).

As insects cannot produce sterols, they need to obtain it from their diet. Cholesterol and demesterol are crucial for development, as without it, larvae cannot progress past the first instar (Wigglesworth, 1953).

3.1.2.3 Nucleic acids and sugar

A lack of nucleotides in the larval diet results in extremely slow growth. Usually, larvae that are not provided with dietary nucleotides have a maximal eclosion rate of 5%. RNA is usually derived by the hydrolysis of yeast in the highly alkaline gut of the larvae (Clements, 2000). Sugar, however, is not essential for larval growth, and can be replaced by high concentrations of amino acids. The presence of glucose does result in an increased rate of growth, but only to certain concentrations of sugar (Clements, 2000).

3.1.3 The biology and endocrinology of the fat body

The principle organ of nutritional storage in both the adult and immature mosquito is the fat body. The fat body exists as a sheet that adheres to the body cavity throughout the thorax and abdomen. In well nourished larvae the fat body is packed with lipid droplets, and contains glycogen deposits and protein vacuoles. The three types of protein vacuole that exist in eclosion disappear shortly after eclosion (Wigglesworth, 1953). Well-fed larvae that are starved deplete the lipid stores in the fat body, then go to other glycogen reserves such as the posterior portions of the stomach, muscle sarcoplasm and central nervous system (Wigglesworth, 1953).

Larval starvation has a marked effect on the structure of the fat body. Nutrient deprivation results in rapid reorganisation of the fat body. Stored metabolites are depleted, and there is a rapid loss of mitochondria and rough endoplasmic reticulum. This situation can be reversed by the re-introduction of nutrients (Liu *et al*., 2009). If the nutritional reserves are not replenished, the emergent adult will suffer from various disadvantages.

It is already well established that larvae that do not receive optimal nutrition will be smaller than their well-fed counterparts (Briegel, 1990). Small, malnourished mosquitoes have an altered endocrinology, particularly in reference to the synthesis of juvenile hormone. There is a strong correlation between teneral reserves (the reserves acquired during larval feeding) and the activity of the Corpora Allata (CA) gland (Noriega, 2004).

The achievement of a critical weight that sets the final larval instar on a commitment to metamorphosis is a hallmark of holometabolous insects (Nishiura *et al*., 2007). During this physiological transition, activity of the CA is reduced, resulting in a reduced concentration of Juvenile Hormone (JH). A subsequent increase in ecdysone titre will initiate metamorphosis (Shapiro *et al*., 1986). The nutritional condition of the fourth instar larvae is crucial for this transformation, and as such, this is the stage in the aquatic life where the larvae feed most vigorously. Appropriate nutritional stores are required for the attainment of critical weight and subsequent commitment to pupation (Telang *et al*., 2007). The endocrinological sequence of events that happen after pupation are well described for *Ae. aegypti*. Although this has not been well mapped in malaria vectors, there is enough evidence to suggest that the mechanisms are very similar. Once critical weight is achieved, neutral lipid stores from the midgut are depleted in an apparent JH-controlled reaction (Nishiura *et al*., 2007). Juvenile hormone levels are low at eclosion, and have a sharp subsequent peak after emergence, usually within 24 hours (Shapiro *et al*., 1986). This peak in JH synthesis and activity is due to CA activity that is promoted by sufficient nutritional reserves. Inadequate larval reserves prevent this peak in JH activity (Caroci *et al*., 2004).

Not only does nutrient concentration have a reproductive effect via the modulation of JH, but the presence of larval fat cells is crucial for ovarian development. 50% of nutrients in the young *Drosophila* female's ovary are derived from larval carbon sources. This is dictated by a strict programme of apoptosis in the larval fat cells which determine larval resource allocation in the adult (Aguila *et al*., 2013). This all demonstrates that larval feeding has a critical effect on adult female reproduction, and consequently, vectorial capacity.

Although adult reproductive capacity is inhibited by inadequate larval reserves, a variety of adult morphological traits are affected by larval resource allocation, not only in mosquitoes, but holometabolous insects in general (Boggs and Freeman,2005). The type of starvation is just as important as the starvation itself. In the case of the forest tent caterpillar *Malacosoma disstria*, a carbohydrate based diet results in resource allocation to somatic growth, while a standard or protein rich diet results in more commitment to reproductive function (Colasurdo *et al*., 2009).

Several important life history traits that affect vectorial capacity are affected by larval nutritional history. The most important of these is longevity. Several studies have demonstrated that larval nutrition affects subsequent adult longevity in several species. These include the butterfly *Spergia mormonta* (Boggs and Freeman,2005), *Anopheles arabiensis* (Ameneshewa and Service, 1996), *An. gambiae* (Lehmann *et al*., 2006) and *An. largini* (Araujo *et al*., 2012). This is crucial as small changes in longevity have a large effect on vectorial capacity (Garrett-Jones and Shidrawi,1969). Furthermore, an increase in food quality at the larval stage not only increases longevity, but also results in increased biting frequency and blood-meal length (Araujo *et al*., 2012).

3.1.4 Larval nutrition and immunity

The major concern about how environmental effects will affect mosquitoes pertains to their vectorial capacity. Although all the factors discussed beforehand would play a role in vectorial capacity, the single most important factor determining this capacity is the vector's immune system. In relation to this study, the major question remains: will larval nutrition have an effect on the capacity of adult females to transmit plasmodia?

To address this issue broadly, it is important to note that in adult insect's nutritional conditions, specifically short term dietary deprivation has a marked effect on immune function. As early as 1963 it was noted that the ability to respond to immune insult by melanin production was a nutrient mediated effect (Sang and Burnet,1963). Studies of the red flour beetle *Tenebrio molitor* demonstrated that a short term nutrient deprivation of the adult beetle had a negative effect on immune function. This could be corrected by the re-introduction of a standard diet (Siva-Jothy and Thompson, 2002).

Larval nutrition also plays a role in immunity. When dietary intake is increased, *Drosophila melanogaster* larvae are better able to withstand parasitoid wasp attack. This is due to increased melanisation capacity (Vass and Nappi,1998). In the case of mosquitoes, the question asked is how does the modulation of the immune system due to larval nutrition affect vector competence?

At present, there is no conclusive answer. There are arguments for both small (low teneral reserve) and large (high teneral reserve) individuals making better vectors. The biggest issue is whether smaller or bigger mosquitoes contained more parasites. It has been shown

that larger *An. gambiae* have fewer sporozoites than their medium-sized counterparts (Lyimo and Koella, 1992). It has also been shown that smaller mosquitoes have more free parasite, as they are less capable of melanising them (Suwanchaichinda and Paskewitz, 1998). This observation also holds true for *Plagiorchis elagans* infection of *Ae. aegypti*, where bigger mosquitoes have a smaller parasite load (Mitchell-Foster *et al*., 2012). In contrast, another study showed that poor larval diet reduced the prevalence and intensity of *P. yoelii nigeriensis* infections in *An. gambiae* and *An. stephensi*. The smaller *An. gambiae* suffered from high mortality due to their infections (Takken *et al*., 2013). Furthermore, it has been suggested that smaller mosquitoes will require more blood-meals to complete their gonotropic cycles, thus increasing their contact with humans and thus their vectorial capacity (Shiao *et al*., 2008). Alternatively, it is established that larger mosquitoes live longer (Ameneshewa and Service, 1996 , Lehmann, 2006), and will bite more frequently and take longer bloodmeal duration (Araujo *et al*., 2012), which in turn would also increase their vectorial capacity. In summary, there is no conclusive argument on the effect of larval nutrition on parasite transmission.

3.1.5 Agriculture and malaria transmission

Agricultural activities play a crucial role in malaria transmission. Ijumba and Lindsay (2001) coined the term "the paddies paradox". This refers to the phenomenon that irrigated areas have reduced cases of malaria. The proposed explanation for this is that the less efficient malaria vector, *An. arabiensis*, displaces the highly efficient *An. funestus* in rice fields (Ijumba and Lindsay 2001). Further studies of rice-growing areas confirm that the dynamics of malaria transmission are affected largely by a change in species composition in agricultural areas, and more specifically the effects of agriculture on *An. arabiensis* (Muturi *et al*., 2008b).

A study in Burundi indicated that the vectorial capacity of *An. gambiae s.l.* (predominantly *An. arabiensis*) is 150% greater in rice irrigated schemes rather than cotton agricultural schemes (Coosemans, 1985). It is generally agreed that planned agriculture lowers *An. funestus* transmitted malaria, but not *An. arabiensis*-borne disease. This may be largely due the high densities of *An. arabiensis* in these areas and the relative scarcity of *An. funestus* (Muturi *et al*., 2008a). This may be because *An. funestus* is only abundant in rice growing areas when the crop is mature, while *An. arabiensis* predominate when the crop is

immature (Githeko *et al*., 1996). More specifically, *An. arabiensis* predominate over the three week period after transplantation. Larval density of this species also increases with the application of fertilizer. *Anopheles arabiensis* are found at low densities during the late vegetative, reproductive and ripening phases. They are subsequently replaced by *An. funestus* (Mwangangi *et al*., 2006).

Maize agriculture has an even greater effect on malaria transmission than rice agriculture. It was demonstrated that maize cultivation is associated with malaria transmission in the Bure district of Ethiopia. This increase is due to *An. arabiensis*-borne transmission, rather than a change in population dynamics (Kebede *et al*., 2005). It is generally agreed that the increased success of *An. arabiensis* in maize growing areas is due to the abundance of maize pollen. Maize (*Zea mays*) pollen is large and serves as an excellent nutritional source for *An. arabiensis* (Ye-Ebiyo *et al*., 2000). Maize pollen is often found abundantly where *An. arabiensis* larvae breed. The larvae readily ingest the pollen which promotes their growth and development. Larvae grow most rapidly when the maize is flowering (Ye-Ebiyo *et al*., 2000). Maize is such a good food source that excess maize pollen can compensate for larval crowding (Ye-Ebiyo *et al*., 2003). Maize is often grown near households, and as such, the enhanced mosquitoes can participate in the malaria cycle close to human hosts (Kebede *et al*., 2005, Ye-Ebiyo *et al*., 2000).

Maize pollen is large (50-90µm) and thin-walled (Raynor *et al*., 1972). Maize pollen can be distributed between 100-200 metres (Luna *et al*., 2001). Rice (*Oryza* spp.) and maize are graminoid, monocotyledonous herbaceous grasses that are wind pollinated. Rice pollen is not as efficiently dispersed as maize pollen, with a mean dispersal distance of 38.4 metres (Song *et al*., 2004). Grass pollen is efficiently distributed and at such high concentrations that they are common allergens (Sen *et al*., 2003). In contrast, cotton (*Gossypium* spp.) is a crop that tends to be bee pollinated. The flight range of bees is generally short, around 25 metres (Pierre and Hofs, 2010), and as such cotton pollen is not as freely available in the environment. Consequently, cotton pollen is not as available as a larval food source, possibly explaining why maize and rice agriculture result in increased vectorial capacity of *An. arabiensis* compared to cotton agriculture (Coosemans, 1985).

3.1.6 Nutrition, insecticide resistance and study rationale

It is clear that larval nutrition is a crucial component of adult vectorial capacity of various mosquito species. There is, however, an obvious fitness effect that would play a role in vectorial capacity that has not been examined and that is insecticide resistance. Prior to this study, no previously published work existed concerning the effect of larval nutrition on the insecticide resistant phenotype. The question to be asked is whether this is due to a lack of research or because there is no evidence that there would be an effect on insecticide resistance.

The answer appears to be a lack of research. A small body of research exists on diet and insecticide resistance. Adult houseflies, Musca *domestica vicinia*, raised on goat's milk are more resistant to DDT than those raised on cow's milk or other diets. *Aedes aegypti* larvae raised on a diet of milk are slightly more susceptible to insecticides than those raised on wheat flour (Gordon, 1961). The molecular and biochemical mechanisms for this in insects are completely unexamined, but studies in mammals such as rats provide a clue. Phase I and Phase II detoxification reactions are strongly affected by nutritional components. This is summarised in Figure 3.1.1. Niacin, riboflavin, pantothenic acid and pyridoxine are crucial for phase I reactions. Phase II reactions are also dependent on nutritional requirements (Rose and Hodkingson, 2004). It is therefore conceivable that nutritional factors may play a role in the detoxification reactions of invertebrates.

The aim of this study was therefore to assess whether larval nutrition has an effect on the expression of the insecticide resistant phenotype of laboratory reared *An. arabiensis*. If there is an effect, the next question is how this is mediated? Is it due to vigour tolerance, a change in neuronal sensitivity to insecticides, a change in detoxification enzyme activity or a combination of these factors?

This study uses two genetically related strains which differ in that one was selected for the expression of insecticide resistance. Unlike a study using wild material, there are less confounding factors about to their differing responses. As the insecticide resistant strain was selected from the susceptible base strain, they are largely genetically homogenous, and the effects can be more strongly attributed to environmental factors.

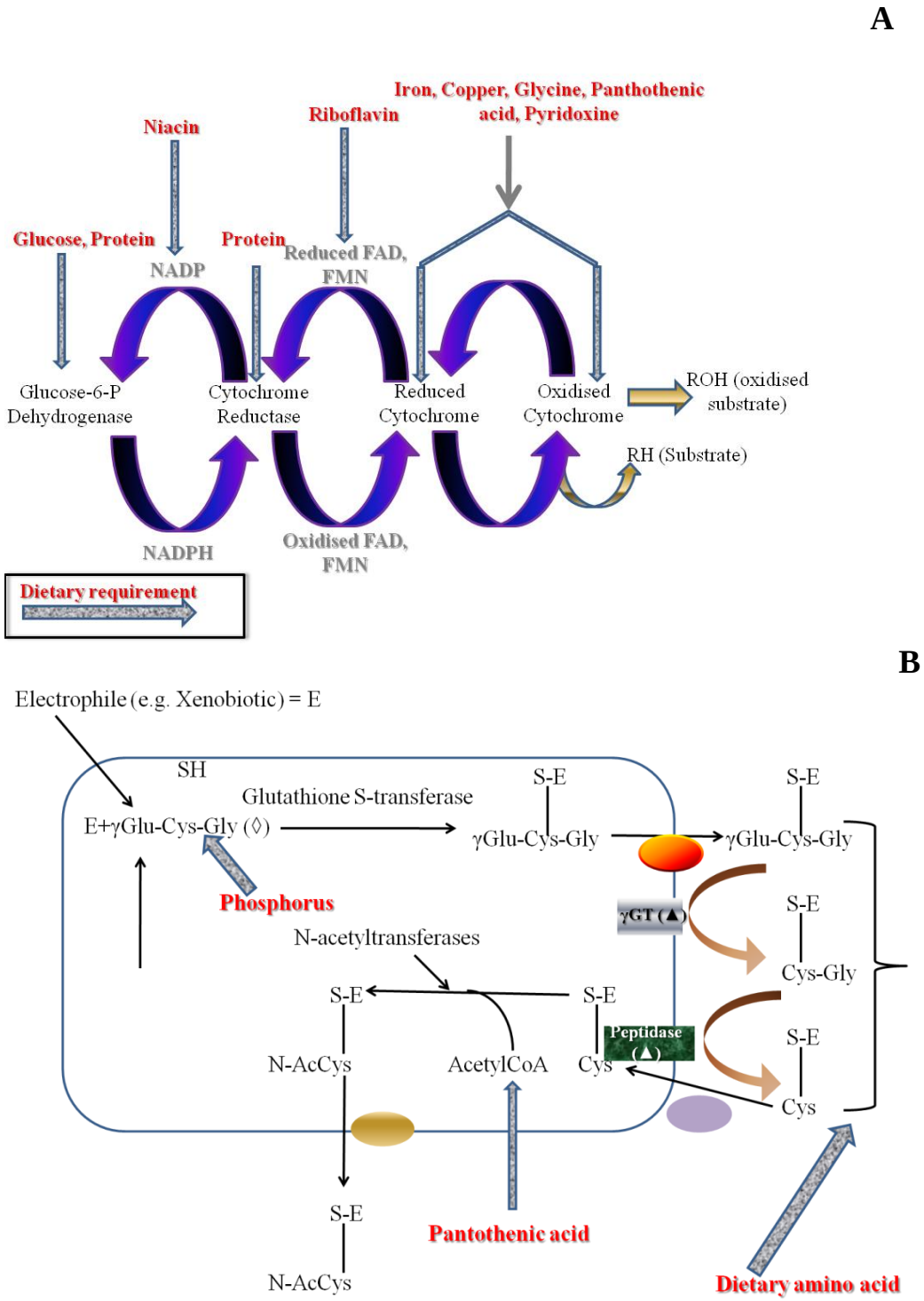


Figure 3.1.1: Nutritional requirements for detoxification reactions. A: requirements for the Cytochrome P450 reactions, an archetypal Phase I reaction. B: nutritional requirements during the formation of mercapturic acid, an archetypal GST-mediated Phase II reaction. The glutathione s-conjugate (◊) is produced intracellularly and transported out of the cell for degradation by ectoproteins (▲). The cysteine conjugate is transported back into the cell and N-acetylated to form mercapturic acid. Ac: Acetyl, CoA: Co-enzyme A, Cys: cysteine, Glu: glutamate, Gly: glycine, S-E: Thiol conjugate, SH: sulfhydryl group. Figure adapted from Rose and Hodgkinson (2004) and Wang and Ballatori (1998) (Rose and Hogkinson, 2004; Wang and Ballatori, 1998).

3.2 Materials and Methods

3.2.1 Mosquito strains used

The *Anopheles arabiensis* strains SENN and SENN DDT were used in this study. As members of the *An. gambiae* complex, they naturally breed in temporary bodies of water and are likely to be subjected to more fluctuating access to food resources than members of the *An. funestus* group that breed in permanent and semi-permanent bodies of water. A description of the housing and rearing conditions of these stains is given in Section 2.2 and Chapter 2 describes the biology of resistance mechanisms of the resistant SENN DDT strain.

3.2.2 Larval fasting regimes (Generalised)

Larval fasting regimes were set up as follows: For every experiment, randomly selected hatchlings less than 24 hours were picked and placed into a set volume of water depending on experiment (see below). Larvae were selected from a single egg batch per experiment. Larvae were exposed to one of four fasting regimes, differing in frequency of feeding. All treatments were fed a total of 1mg of food per larva. The daily cohort (1 day) was fed once each day. The two day cohort (2 day) was fed once every second day and the three day cohort (3 day) were fed once every three days. The control cohort was fed three times daily, every day.

3.2.3 Development studies

Both the SENN and SENN DDT strains were used for this experiment. Each experiment consisted of 50 larvae per 300ml distilled water. Each regime was therefore fed 50mg of food per feeding session as described in 3.2.2. Ten replicates were performed for each cohort, and for each replicate the larval fasting regimes were performed in parallel.

The development of the larvae was monitored daily. Two developmental milestones were recorded: the emergence of the first pupae, and the day 50% or more of the pupae emerged on a single day. This was referred to as the pupal burst or maximal emergence day.

The mean time until day of first pupal emergence and mean maximal emergence time between the different larval feeding regimes were compared using a 1-way Analysis of

variance (ANOVA), using a Tukey's test as a post hoc test. The confidence interval was set at 95%.

3.2.4 Determination of adult susceptibility to DDT

Both the SENN and SENN DDT strains were used in this experiment. To obtain adults for the study, a 2 day cohort was set up. A cohort of 200 hatchlings (each less than 24 hours old) of each strain was placed into 1200ml of distilled water. For each growth cycle, 5 replicates of the described fasting regimes were set up to develop concurrently. For each bowl, the larvae of the two day cohort were fed 200mg of food once a day on alternating days. The feeding regime of the control cohort was set up in the same manner, except that this cohort was provided with 200mg of food three times a day on a daily basis.

Upon eclosion, adults of the same strain, fasting regime and age were collected, kept together and provided with a 10% sucrose solution until the age of 3 days. Males and females of the same age were allowed to share a cage and mate, but females were not offered a blood-meal during their lifetimes. Examples of female and male mosquitoes are represented in figure 3.3.3A and B respectively.

At the age of 3 days, the adults were bioassayed using the CDC bottle bioassay (Brogdon *et al*., 1998). The insides of 200ml glass bottles were coated with 1ml of 1mg/ml DDT (Sigma Aldrich, 31041) with acetone as a carrier solution. The bottles were allowed to dry overnight. For the DDT susceptible SENN strain, bottles were used 5 times before discarding, while for the resistant SENN DDT strain bottles were used twice before discarding.

Lethal times were determined for both strains. Time was used rather than concentration, as the lethal concentration could not be determined for SENN DDT since the concentrations required to obtain 50% mortality on the resistant strain would not remain in solution. Twenty 3-day old, non blood-fed adults (either male or female with the sexes kept separately) were used per bottle. The adults were exposed to DDT for varying lengths of time. SENN adults were exposed for either 2.5, 5, 10, 15, 20, 25 or 30 minutes. SENN DDT adults were exposed for 2, 4, 8 and 16 hours. During the exposure periods, the adults were not allowed access to sugar, but after the exposure period, they were removed and allowed to rest in cups where they were allowed access to sugar. Mortality was scored 24

hours post exposure. Control exposures were performed by an exposure to an acetone coated bottle for the same period of time. This constituted a handling control, ensuring that any mortality observed in the treatments could not be attributed to the movement of mosquitoes between containers. An environmental control consisted of unexposed mosquitoes remaining in a cup, without access to sugar, for the duration of the exposure. This ensured that any mortality observed was not due to environmental factors. A total of 50 replicates per time period was performed for SENN (n=7000), and a total of 25 replicates per time period was performed for SENN DDT (n=2000). The mortality data were plotted against the log value for each time period and a linear regression was performed to determine the lethal time (LT). The LT_{50} for SENN DDT and the LT_{75} for SENN was determined. This was because an LT_{50} could not be accurately determined for SENN.

3.2.5 Determination of adult size

All experiments subsequent to the determination of adult DDT susceptibility were to determine why any changes in phenotype occurred. None of the following experiments were performed on the mosquitoes used to determine lethal time. For each subsequent experiment, a new fasting regime, as described in 3.2.4, was set up as only 2 day cohorts were used as a fasting regime. This was due to the difficulty in obtaining adequate numbers of viable adults for experiments from the 3 day cohorts.

The following experiment was performed to determine the importance of vigour tolerance in the response of fasted adults. Wing length can be used as an approximation of adult size as it correlates well with dry weight (Nasci, 1991). The sizes of SENN adults that survived a 2.5 minute exposure to 1mg/ml DDT, as described in 3.2.4, were compared to those that had succumbed in the same period. The sizes of SENN DDT adults that survived 16 hours exposure were compared to those that had succumbed in the same period. For each strain, adults from the control feeding regime were also exposed to the same treatment and the sizes of the survivors and the dead were compared. To ensure that no changes could be attributed to shrinkage that happened overnight, adults that were confirmed dead immediately after the exposure period were used. Survivors were collected at the same time and ether killed. Mosquitoes were exposed to cottonwool soaked in diethyl ether until they succumbed to the fumes.

Wing length was determined by the method described by Lyimo *et al.* (1992). Fifty individuals were used for each strain and gender. Both wings of each individual were fixed to a glass slide. The wing length was determined using a calibrated micrometer at 200X magnification. The distance from the distal end of the alula to the tip, excluding fringe scales was determined (Lyimo *et al*., 1992). A two sample t-test was used to determine whether a significant difference in size between the survivors and those that succumbed to DDT existed. The confidence interval was set at 95%.

3.2.6 Ocurrence of *kdr* mutations in association with *An. arabiensis* laboratory strain

Although Matambo *et al.*(2007) described the *kdr* status of SENN DDT, the status of both SENN and SENN DDT was re-examined, as this study took place 6 years later. An exposure was performed as described in 3.2.4. The *kdr* genotype of the survivors and those that succumbed was determined by hydrolysis probe assay, as described by Bass *et al.*(2007). This method is detailed in chapter 1.

3.2.7 Detoxification enzyme activity in association with larval nutrition

The SENN and SENN DDT strains were used in this experiment to determine the effect of larval nutrition on the activity of metabolic detoxification enzymes. Strains for this experiment were prepared as described in 3.2.4, i.e. 2 day feeding regimen cohorts compared to control cohorts.

For each strain, gender and feeding regime, fifty 3-day old adults that had not been blood fed were assayed. The enzyme activities of 3-day old adults from the susceptible *An. arabiensis* strain KGB were used as a baseline comparison. The absolute activities of the Cytochrome P450, Glutathione S-transferases and general esterases were determined as described in Section 1.2.3.

3.3 Results

3.3.1 Larval nutrition and development

As expected, larval development rate is inversely proportional to the amount of larval nutrition. Control larvae developed the fastest, with the 3 day cohort developing the slowest. For SENN DDT only the 3 day cohort developed significantly slower. The first

pupae emerged significantly later than the control (2 sample t-test: $p<0.01$, $t=-18.67$, $df=17.6$) as was the case for maximal emergence time ($p<0.01$, $t=-35.67$, $df=15.7$). For SENN the 3 day cohort emerged significantly later than the control ($p<0.01$, $t=-23.67$, $df=18$), as did the 2 day cohort ($p<0.01$, $t=-8.76$, $df=18$). Although the 3 day cohort had a significantly delayed maximal emergence ($p<0.01$, $t=-23.26$, $df=15.8$), the 2 day cohort did not have a significantly delayed maximal emergence (Figure 3.3.1).

When comparing the two strains, the SENN control cohort developed significantly faster until the point of first pupal emergence than control SENN DDT ($p<0.01$, $t=6.48$, $df=18$). There was, however, no significant difference in the time of maximal emergence. There was no significant difference in time to first emergence of pupae or maximal emergence time between SENN and SENN DDT in the 2 and 3 day cohorts (Figure 3.3.1).

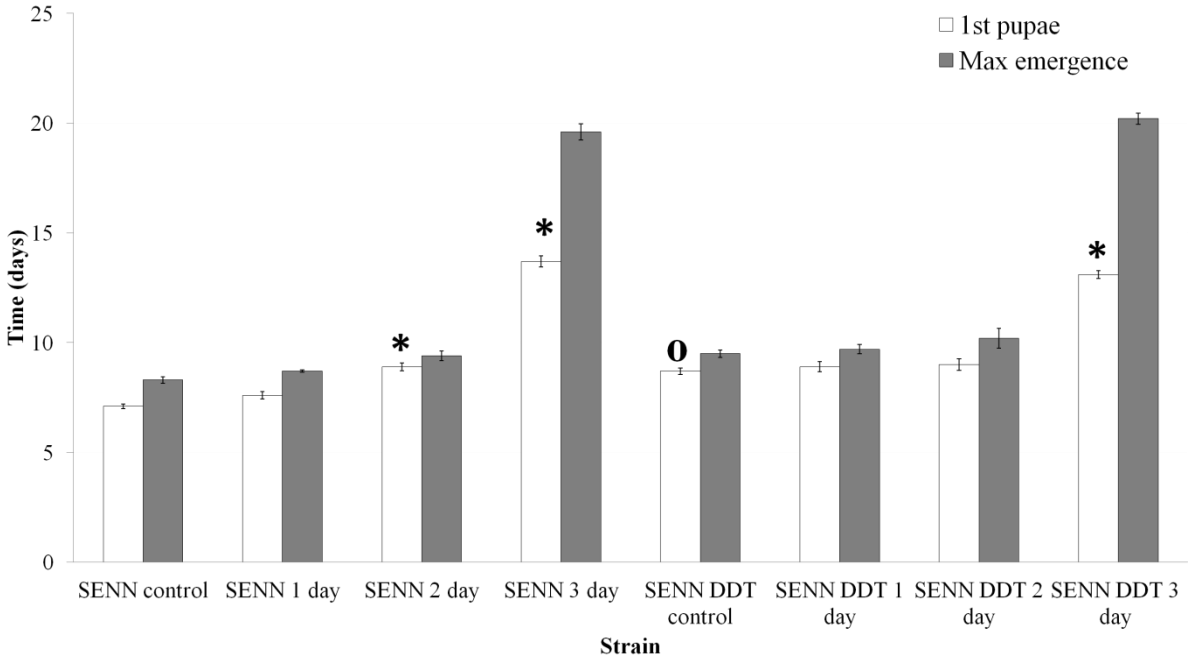


Figure 3.3 1: Larval development times (expressed as days to first pupations and days to 50% pupation) by larval feeding cohort for the *Anopheles arabiensis* SENN and SENN DDT laboratory colonies. Cohort feeding regimes were thrice daily for the controls, once daily (SENN and SENN DDT daily), once every second day (SENN and SENN DDT 2nd day) and once every third day (SENN and SENN DDT 3rd day). A total of 500 larvae per treatment were monitored, divided into 50 larvae per replicate. Asterisks (*) indicate a significant difference from the time of the first appearance of pupae in the control group of the same strain. A circle (o) indicates a significant difference in the time of first emergence of pupae in the control groups of SENN and SENN DDT.

3.3.2 Larval nutrition and the DDT resistant phenotype

Larval nutrition had a marked effect on the expression of DDT resistance. Fasted individuals of the susceptible SENN strain showed a significant reduction in lethal time compared to control individuals for both males (2 sample t-test: $p < 0.01$; $t = 5.52$) and females ($p < 0.01$; $t = 13.51$) of the strain. There was no significant difference between the lethal times of control males and females, and that of the fasted males and females. The results are presented in Block A of figure 3.3.2.

The results are not directly mirrored by the resistant SENN DDT strain. While there was a significant decrease in the lethal time of the fasted SENN DDT females ($p = 0.02$, $t= 3.54$) compared to the control strain, there was no significant difference in the lethal times of fasted and control males. There was a significant difference between the lethal times of the control males and females (2-sample t test: $p = 0.02$; $t = 3.54$), and the lethal times of the fasted males and females ($p = 0.02$; $t = 3.74$). . The results are presented in Block B of figure 3.3.2.

3.3.3 Biological basis for altered phenotype

From the lethal time studies, it is clear that the larval nutrition has a marked effect on the DDT resistant phenotype. The remainder of the study examined the biological basis of this phenotypic change. Three factors were examined. The first was to determine whether mosquitoes that succumb to DDT intoxication are always smaller, thereby determining the importance of vigour tolerance. The second was to confirm the *kdr* genotype of the mosquitoes. Finally, the activity of the metabolic detoxification enzyme families (Glutathione s-transferases, Cytochrome P450s and general esterases) were quantified to determine whether fasted mosquitoes had an altered rate of detoxification enzyme activity.

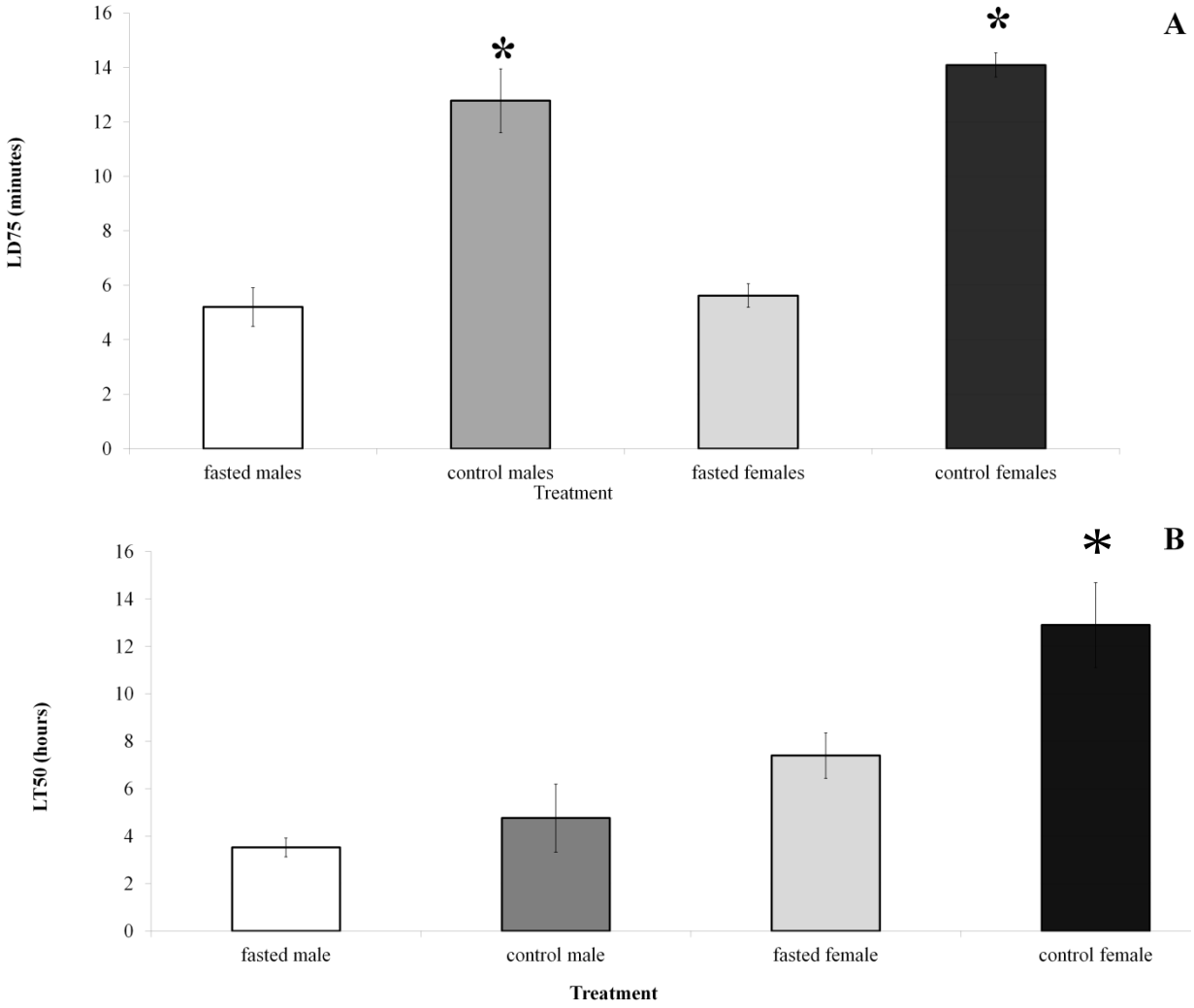


Figure 3.3.2: (A) Mean lethal times (minutes) required to induce 75% mortality (LT75) in adult SENN *Anopheles arabiensis* by exposing them to DDT at a concentration of 1mg per 250 ml glass bottle. Adults eclosed from fasted larvae (fed once every second day) are compared to adults eclosed from control larvae (fed thrice per day) by gender. (B) Mean lethal times (hours) required to induce 50% mortality (LT50) in adult SENN DDT *Anopheles arabiensis* by exposing them to DDT at a concentration of 1mg per 250 ml glass bottle. Adults eclosed from fasted larvae (fed once every second day) are compared to adults eclosed from control larvae (fed thrice per day) by gender. Asterisks (*) indicate a significantly different increase it lethal time compared to the fasted equivalent.

3.3.3.1 Vigour tolerance

Vigour tolerance does appear to play a role in the phenotypic responses of the strains, but to a differing extent. Fasted mosquitoes were always smaller than their control counterparts (ANOVA females: $p < 0.01$; $F = 28.4$; males: $p < 0.01$; $F = 61.1$; $df = 199$) (figure 3.3.3).

An example of the effect of nutrition on size is displayed in figure 3.3.3. When comparing the size of survivors and those that succumbed to DDT, the SENN survivors were always larger than those that succumbed (ANOVA: $p < 0.01$; $F = 40.2$; $df = 199$), as seen in figure 3.3.4. This is not mirrored in the SENN DDT strain. There was no significant difference between the survivors and those that succumbed (ANOVA: $p < 0.01$; $F = 9.05$, $df = 199$). The only exception was the control female survivor cohort in which individuals were significantly larger than those that did not survive DDT treatment ($p=0.03$). These results are summarised in figure 3.3.5.

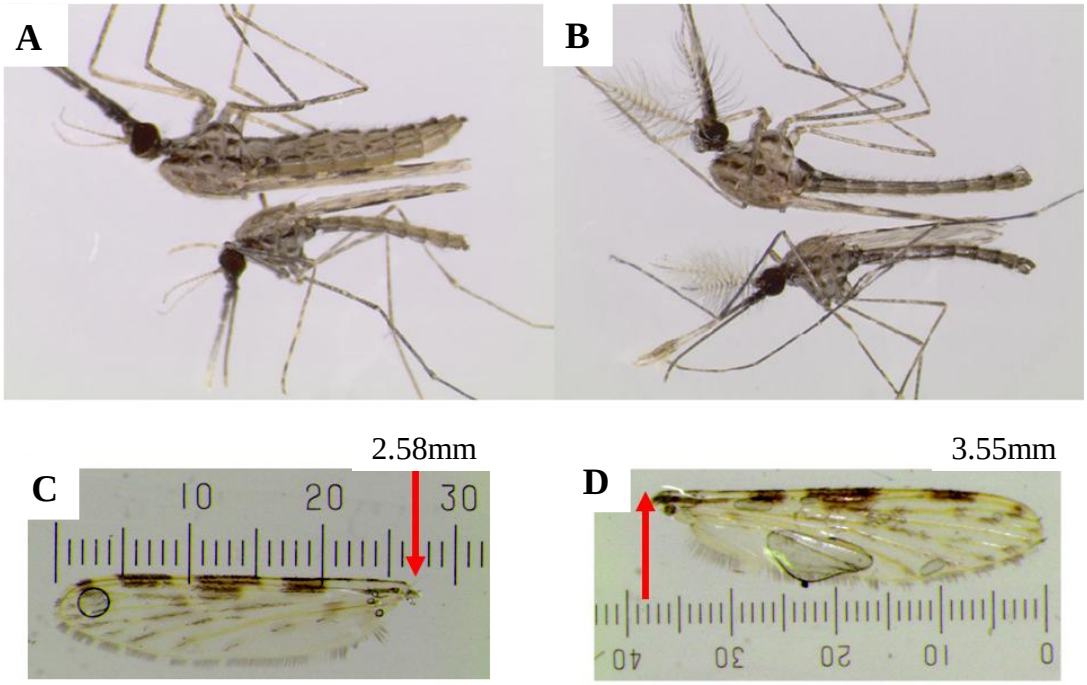


Figure 3.3.3: The effect of larval nutrition on the size of SENN DDT adults. A (females) and B (males): Adults eclosed from 2-day fasted larvae (represented at the bottom of the pairs) are compared to their control counterparts of the same age. C: Measurement of wing of fasted female. D: Measurement if wing of control female. The graduations of the micrometers is 0.01 of an inch.

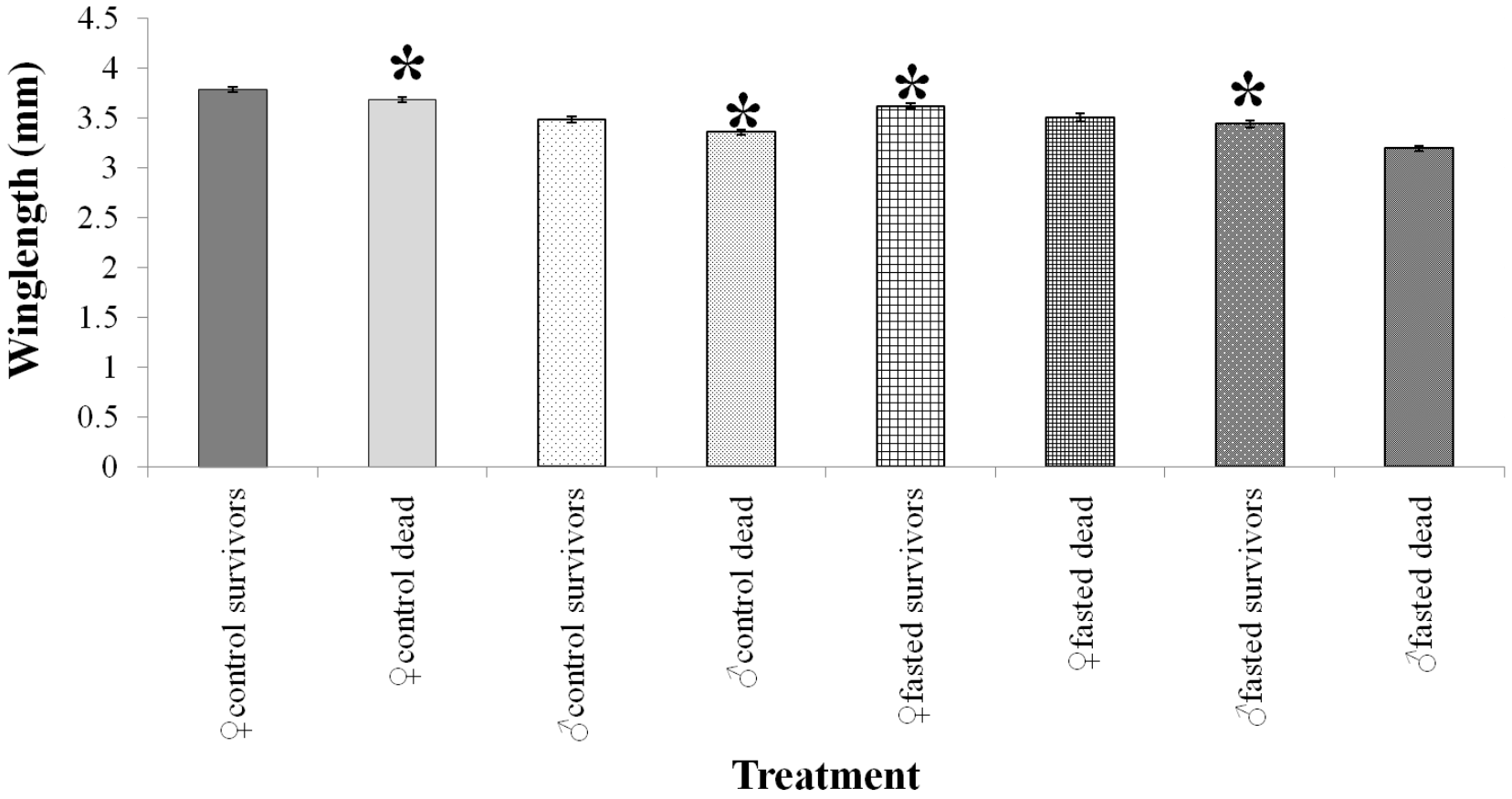


Figure 3.3.4: Wing lengths (mm) of *Anopheles arabiensis* SENN survivors and susceptibles (dead) following a 2.5 minute exposure to 1 mg/ml DDT. Control cohorts (eclosed from larvae fed thrice daily) are compared to their corresponding fasted cohorts (eclosed from larvae fed once every second day) by gender. Asterisks (*) denote significant differences between survivors and dead.

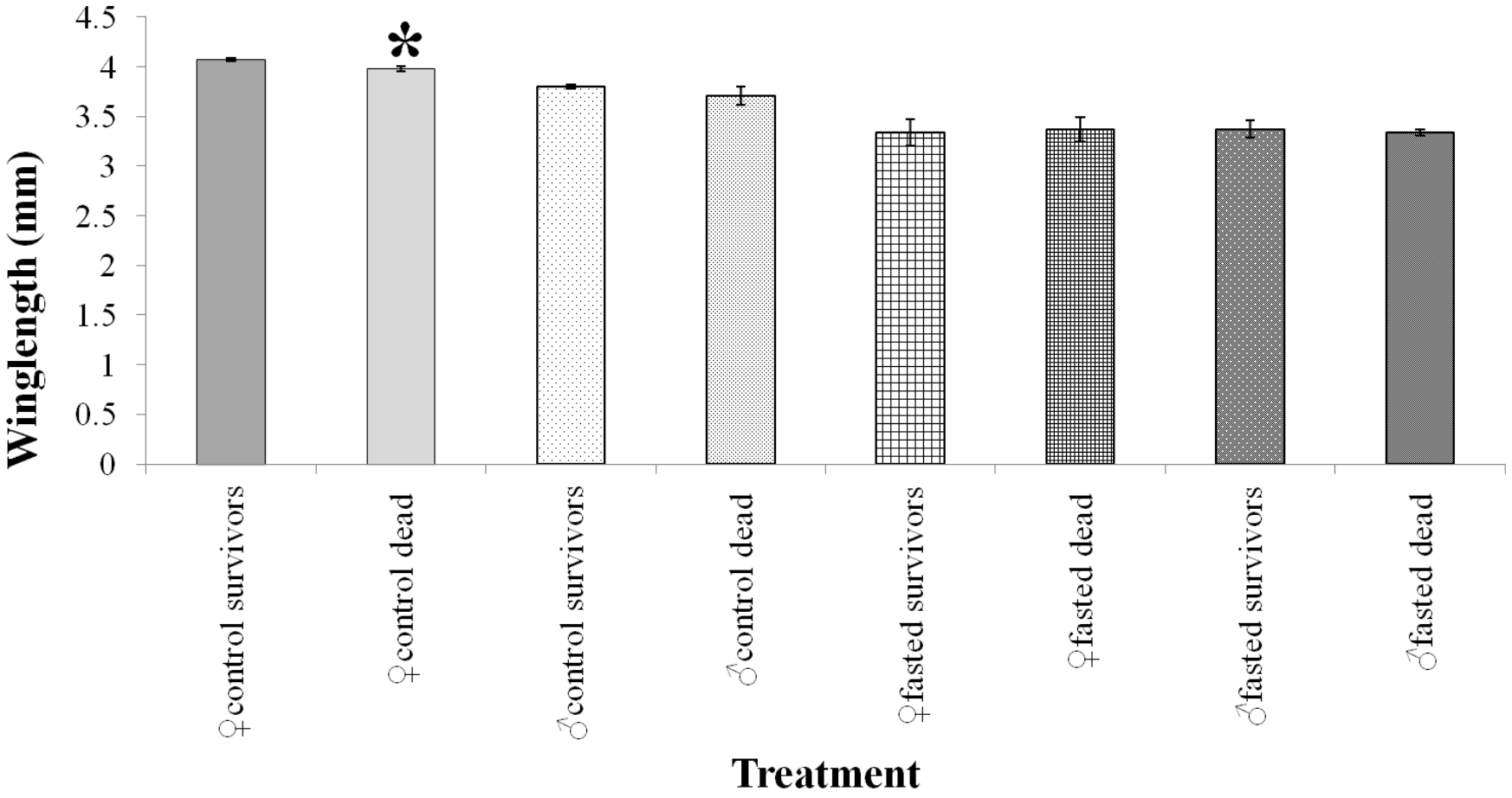


Figure 3.3.5: Wing lengths (mm) of *Anopheles arabiensis* SENN DDT survivors and susceptibles (dead) following a 16 hour exposure to1mg/ml DDT. Control cohorts (eclosed from larvae fed thrice daily) are compared to their corresponding fasted cohorts (eclosed from larvae fed once every second day) by gender. Asterisks (*) denote a significant difference between survivors and dead.

3.3.3.2 *kdr* genotyping

The *kdr* genotype of DDT-exposed SENN and SENN DDT was determined by hydrolysis probe assay. All individuals from the SENN strain were homozygous susceptible for both the L1014F and L1014S mutations. Although all SENN DDT individuals were homozygous susceptible for the L1014S mutation, they were all homozygous resistant for the L1014F mutation.

3.3.3.4 Metabolic detoxification enzyme activity

As observed in Chapter 2, control SENN DDT individuals showed significantly higher levels of detoxification enzyme activity than that of the standard susceptible *An. arabiensis* strain KGB for cytochrome P450 (ANOVA: $p < 0.01$, $F = 19.1$ for females; $p < 0.01$, $F = 22.7$ for males; df = 489), GST (ANOVA: $p = 0.02$, $F = 4.28$ for females; $p < 0.01$, $F = 15.4$ for males; df =489), α-Esterase (ANOVA: $p < 0.01$, $F = 8.79$ for females; not upregulated in SENN DDT males; df = 489) and β-esterase (ANOVA: $p < 0.01$, $F = 9.89$ for females; $p < 0.01$, $F = 25$ for males; df = 489).

A general decrease in metabolic enzyme activity was observed in all fasted strains. Table 3.3.1 summarises the mean activities of the three enzyme classes of the susceptible baseline KGB, the fasted base cohorts (SBF), fasted DDT cohorts (SDF), control base cohorts (SBC) and control DDT cohorts (SDF).

Table 3.3.1: Mean absolute detoxification enzyme activities for the *Anopheles arabiensis* SENN and SENN DDT strains by larval feeding regime cohort by gender

	Cytochrome P450 equivalents/mg protein (SD)	α Esterase - α napthol equivalents/mg protein (SD)	β Esterase - β napthol equivalents/mg protein (SD)	Glutathione S-transferase µmol/min/ml (SD)
KGB♂	0.0046 (0.0012)	0.0037 (0.0011)	0.0044 (0.0008)	0.000072 (0.00001)
KGB♀	0.0053 (0.0017)	0.0048 (0.0013)	0.0055 (0.0017)	0.000103 (0.000041)
SBF♂	0.0047 (0.0018)	0.0013 (0.0008)	0.0049 (0.0016)	0.000046 (0.00002)
SBF♀	0.0047 (0.0014)	0.0017 (0.0007)	0.0048 (0.0013)	0.000048 (0.000017)
SBC♂	0.006 (0.0018)	0.0017 (0.001)	0.0061 (0.0021)	0.000057 (0.00003)
SBC♀	0.0063 (0.0015)	0.0039 (0.0017)	0.0052 (0.0015)	0.00007 (0.000072)
SDF♂	0.0105 (0.0034)	0.0012 (0.0006)	0.0038 (0.0006)	0.000109 (0.0001)
SDF♀	0.0081 (0.0027)	0.0041 (0.0023)	0.0063 (0.003)	0.000121 (0.000034)
SDC♂	0.0094 (0.003)	0.0014 (0.0007)	0.0077 (0.0014)	0.000139 (0.000066)
SDC♀	0.0102 (0.0035)	0.0066 (0.0034)	0.0068 (0.0011)	0.000144 (0.000041)

Table 3.3.2: Comparison of the statistical differences in enzyme activity of adult An. arabiensis eclosing from fasted larvae to those of non fasted larvae. Dashes indicate no statistical differences, while significant p-values are given in brackets

	SBF♂	**SBF♀**	**SDF♂**	**SDF♀**
P450	↓(0.02)	-	-	↓ (0.02)
α esterase	-	-	↓(0.00)	↓(0.00)
β esterase	↓(0.00)	↓(0.00)	↓ (0.00)	-
GST	↓ (0.02)	-	↓ (0.00)	-

Although there is a general decrease in enzyme activity in both strains, not all the changes are significant. The greatest changes were in Cytochrome P450 activity, significantly decreased in SENN fasted males ($p=0.02$), SENN fasted females ($p<0.01$) and SENN DDT fasted females ($p=0.02$). GST activity was the least affected, with only SENN fasted males demonstrating significantly reduced activity ($p=0.02$). Alpha esterases were significantly decreased in SENN fasted females ($p<0.01$) and SENN DDT fasted males ($p<0.01$) and beta esterases significatly reduced in SENN fasted males ($p<0.01$) and SENN DDT fasted males ($p<0.01$).

3.4 Discussion

This study supports the current body of literature that nutritional restriction at the larval stages has far reaching effects on the development of the larvae and the subsequent life history of the emergent adults. The general observation is that larval fasting generally increases development time and decreases the tolerance of emergent adults to DDT. The effects on development are similar to previous studies, particularly to the study of larval selection in *An. gambiae* conducted by Lehmann *et al.* (2006). This study is unique as it was performed on insecticide resistant and susceptible strains of the sibling species *An. arabiensis*, a generally less-studied species. More importantly, this study was the first published study on the effect of larval starvation on the insecticide resistant phenotype of a mosquito species.

The effect of nutrition on the development of *An. arabiensis* larvae does not differ from any other species. The less food supplied to the larvae, the longer the development time. Typically, the 3 day cohort took double the time of the control groups to pupate. For both strains, there was no significant difference in development rate between controls and those fed once daily. Nutritional stress appears to have a more marked effect on the development of the susceptible SENN strain than the insecticide resistant SENN DDT strain. SENN cohorts fed daily did not differ significantly in development time, but the remaining fasted SENN cohorts developed significantly slower than their control counterparts. In contrast, for SENN DDT only the cohorts fed every three days developed significantly slower (figure 3.3.1).

This is rather surprising. As described in previous chapters, and can be seen by the results in this study, the SENN DDT strain has a higher level of metabolic detoxification activity than SENN. It could be expected that as a result, the strain should be more metabolically active and require more food. From this finding, it may be suggested that if any insecticide resistance-induced fitness cost is present in the strain, the cost is not manifested in developmental rate.

When observing the developmental rate, the control SENN larvae developed significantly faster than their resistant counterparts. This does not necessarily imply a fitness cost in the resistant strain. It is more likely an inadvertent consequence of selection. Early and late hatching *An. gambiae* have been shown to have different insecticide resistant phenotypes (Kaiser *et al*., 2010). Interestingly, in this latter study, late hatching *An. gambiae* had higher levels of DDT resistance than their early hatching counterparts (Kaiser *et al*., 2010). Under standard conditions, SENN DDT tended to hatch later than their susceptible SENN counterparts (unpublished data). This suggests that this result may be a consequence of inadvertent late-hatch selection by virtue of DDT resistance selection, rather than a resistance-induced fitness cost. This idea is further supported by the fact that there was no significant difference in development rate between SENN and SENN DDT strains of the same fasting regime, suggesting that both strains suffer equally from dietary restriction and that the initial assessment that susceptible strains suffer more from larval nutritional deprivation may be more representative of the initial comparison to the fastest developing controls.

The most important finding of this study, however, relates to the insecticide resistance phenotype (figure 3.3.2). The results clearly showed that both male and female SENN adults had significantly reduced DDT tolerance when eclosed from fasted larvae. For SENN DDT, only fasted females had significantly reduced tolerance if they had low teneral reserves. This result is interesting and needs to be dissected as this has implications for vector control.

It is important to note that in SENN adults there was no significant difference in the DDT tolerance of control males and females, and fasted males and females. In contrast, there was a significant difference in DDT tolerance of SENN DDT males and females for both control and fasted groups. As SENN males and females are equally sensitive to DDT, the responses to exposure in both sexes are likely to be equally affected by diet. For SENN DDT, however, it is clear that the larval dietary restriction has a more marked effect on females. This is important as insecticide resistant females provide a particular challenge to vector control efforts. It is also important to consider that a more severe fasting regime, such as the three day fasting regime may have given a different result in SENN DDT males. This will, however, remain speculative. The more extreme fasting regime had too long a development time and a low eclosion rate making it too difficult to obtain sufficient samples for subsequent analysis. The major question to ask, though, is what factors underlie the changes in phenotype that we see in adults that eclose from fasted larvae?

The simplest answer is that the changes are due to an alteration of vigour tolerance. Quite simply, fasted larvae result in smaller adults which require less DDT to be intoxicated. The result of this study reveals that the reality is not that simple. The vigour tolerance hypothesis holds true for the susceptible SENN strains (figure 3.3.4). For all sexes and treatments, adults that died after exposure were always significantly smaller than those that survived. This was not observed in SENN DDT (figure 3.3.5). The results observed in figures 3.3.4 and 3.3.5 are consistent with previous results that females are generally bigger than males (Lyimo *et al*., 1992). In general, there was no significant difference in the size of survivors compared to those that did not survive DDT exposure. The only exception, interestingly, was the case of SENN DDT control females, where survivors were significantly larger than non-survivors. This suggests that vigour tolerance does not have a major effect on insecticide sensitivity in

insecticide resistant adults. The effect is only clearly transduced when there are no other resistance mechanisms at play. Susceptible SENN strains have no target site or metabolic resistance mechanisms; hence the effect of vigour tolerance is very marked. In contrast, SENN DDT adults not only have metabolic resistance, but are also homozygous resistant for the *kdr* mutation. This study shows that these mechanisms appear to play a far bigger role in the resistance phenotype than size, with the exception of control SENN DDT females.

SENN DDT's size, and therefore vigour tolerance, is not the major cause of the phenotypic difference in the resistant strain. Either target site resistance or metabolic resistance is responsible for the changes observed. It is not possible for the *kdr* genotype to be altered in the larvae, therefore either the phenotypic expression of the mutation is affected or GST activity is significantly suppressed. Again, as with vigour tolerance, the answer is not that simple.

The question has been asked whether *kdr* alone can produce a resistance phenotype (Brooke, 2008). This study suggests that this is not the case. The *kdr* genotype cannot be affected by diet, while metabolic enzyme activity is affected, as shown in the detoxification enzyme data. This suggests that enzyme activity does play a role in the SENN DDT strain and that *kdr* is not the sole mediator of DDT resistance in this strain.

In general, larval fasting results in reduced enzyme activity (table 3.3.1). Not all activities, however, are significantly reduced. This may also be the result of using the two day fasted cohort. Although a different scenario may have been observed if the three day cohort was used, this will remain speculative.

It is important to note that Phase I enzymes, cytochrome P450 and esterase were the most affected by diet. Only fasted SENN DDT males did not have a significantly reduced cytochrome P450 activity. All fasted individuals showed significantly reduced esterase activity. Only fasted SENN males had significantly reduced GST activity. This is surprising as GSTs are involved in DDT resistance via DDT dehydrochlorination (Enayati *et al*., 2005). What these data strongly suggest is that insect metabolic detoxification does occur in a phasic manner as do vertebrate pathways (Rose and Hodgson, 2004).

Esterases and cytochrome P450s constitute Phase I detoxification, and involve the derivitivization of substrates making them bioavailable for glutathione tagging by GSTs (Sheehan *et al*., 2001). These results suggest that besides the traditional DDT dehydrochlorination pathway, GST mediated DDT resistance may be a Phase II reaction dependent on the efficacy of Phase I cytochrome P450 activity. Reduced P450 activity may reduce the bioavailability of xenobiotics that can be nucleophilically attacked by GST. This may explain the reduced DDT metabolism in SENN DDT females despite the lack of significantly reduced GST activity. This is further supported by the fact that fasted SENN DDT males only have significantly reduced alpha and beta esterase activity, and as these males do not have significantly reduced P450 activity, that they do not have a significantly reduced concentration of DDT that is derivitized for Phase II GST conjugation. Hence there is no significantly reduced tolerance to DDT.

It is worthwhile noting that these results are not unprecedented. A 1986 study examined the effect of larval diet of the tobacco budworm, *Heliothis virescens,* on the expression of cytochrome P450 and GSTs. Increased diet increased gut microsomal cytochrome P450 activity, indicating the importance of nutrition to cytochrome P450 activity (Riskallah *et al*., 1986).

A study in larval nutrition in *An. gambiae* was published in close proximity to this study. Kulma *et al*..(2013) examined the effect of age and larval nutrition on the DDT resistance phenotype of laboratory reared *An. gambiae.* Although the experimental design and focus of the experiment was different, fundamental similarities were found in both studies. In the Kulma study the resistant Zan/U and the susceptible Kisumu strains were compared. Unlike the present study, these strains are not genetically related. The study concluded that diet did not play a major role in the resistant phenotype. This was because although diet affected the DDT sensitivity of the Kisumu strain, it did not affect the DDT sensitivity of the Zan/U strain (Kulma *et al*., 2013). Again, this may be due to the effect of vigour tolerance, where smaller Kisumu adults, in the absence of other resistance mechanisms, are more affected by slight changes in dosage due to size. In this case, it is important to note that the Zan/U strain only

has increased GST activity (Ranson *et al.*, 2000b) again hinting at the importance of the interplay of Phase I and Phase II reactions suggested by this study.

The ultimate question that should be asked is what is the significance of these findings? Will they ultimately have an operational effect? This can be speculated, but the evidence suggests that it may. This study clearly demonstrates that nutritional deprivation at the larval stage prolongs development and increases sensitivity to DDT. It is the converse that has implications for vector control. It is quite possible that adults with good teneral reserves will present a particular challenge for vector control efforts. This study has implications for increased malaria incidence seen in maize growing areas in particular (Kebede *et al*., 2005).

Varieties of factors, discussed in the introduction, are responsible for the success of *An. arabiensis* in maize and rice growing areas. Insecticide resistance is not often considered one of them. Traditionally, an increase in insecticide resistance associated with agriculture is attributed to poor pesticide management in the agricultural sector, where injudicious use of insecticides can cause resistance in non-target insects (Antonio-Nkondjio *et al*., 2011, Muller *et al*., 2008, Yadouleton *et al*., 2011). Larval exposure to pesticides via runoff may be another selection pressure for resistance (Akogbéto *et al*., 2006).

These data also strongly suggest that the farming of grasses such as rice and maize has an enhancing effect on the fitness of *An. arabiensis* (Kebede *et al*., 2005, Ye-Ebiyo *et al*., 2000). Increased access to larval food generally increases the fitness of anophelines, not only due to a reduced development time, but also results in increased longevity, bite frequency and blood-meal duration (Araujo *et al*., 2012).

As such, it can be expected that larvae that breed near agricultural sites will generally have increased fitness. The effect of this on malaria transmission is still debatable, as are the arguments for both larger and smaller mosquitoes being more efficient vectors. Whichever scenario is correct, it is clear that well fed larvae result in larger, fitter adults that are better capable of expressing an insecticide resistance phenotype. This should be a significant consideration when tailoring vector control strategies.

Chapter 4: The effect of oxidative stress on the life history and expression of insecticide resistance in anopheline vector mosquitoes

4.1 Introduction

4.1.1 Reactive Oxygen Species

Molecular oxygen, the terminal electron acceptor in the process of aerobic respiration, is reduced to water at the end of the oxidative phosphorylation chain. This process, however, is not a single step reaction. In the process of oxygen reduction, reactive intermediates, molecules or atoms with an unpaired electron, are formed. A radical is an uncharged molecule with an unpaired electron (Halliwell, 2006). The radical resulting from the addition of one electron is called the superoxide anion radical, the addition of two electrons results in the formation of hydrogen peroxide, and the addition of three electrons results in the formation of the hydroxyl radical (Freinbichler *et al*., 2011). Oxygen itself, which occurs as a triplet in its'ground state, can be excited to a singlet state, which becomes a highly reactive molecule (Freinbichler *et al*., 2011).

Although oxidative phosphorylation is an efficient process, only 98% of oxygen is fully reduced to water by cytochrome c oxidase. This site is therefore a major source of oxygen radicals. Reactive oxygen species (ROS) are also produced by other enzymes of the oxidative phosphorylation chain. Complexes I and III are particularly leaky sources of ROS (Turrens, 1997). ROS can react within nitric oxide to form Reactive Nitrogen Species (RNS) such as peroxynitrite. Table 4.1.1 gives a summary of various important oxidants. Although this study focuses primarily on ROS, besides reactive RNS, radical (R) and non-radical (NR) chlorine (R: atomic chlorine; NR: Chlorine gas, Chlorine dioxide) bromine (R: atomic bromine; NR: Bromine gas, hypobromous acid) also exist (Halliwell, 2006).

Table 4.1.1: Radical and non-radical reactive oxygen species (Halliwell, 2006, Kohen and Nyska, 2002, Pardini, 1995)

Radical species	Non-radical species
Alkoxyl	Aldehydes
Hydroxyl/perhydoxyl	Ozone
Hydroperoxyl	Hypochlorous acid
Carbonate	Hydrogen peroxide
Carbon Dioxide radical	Peroxynitrite/Peroxynitrate
Peroxyl	Peroxomonocarbonate
Superoxide	Organic peroxides

The half-lives of the major ROS vary greatly, with the most stable of radicals lasting only a few seconds. For particularly unstable radicals such as the hydroxyl radical, the reactions involving the radical take place largely at the site of generation. This is due to the unstable nature of the radical, as it is diffusion limited. More stable radicals, such as some peroxyl radicals, are stable enough to diffuse from their site of origin and are able to cause damage at distant sites (Sies, 1997).

4.1.2 Sources of ROS other than respiration

Although the flavoproteins of the electron transport chain are a major source of ROS, it is not the only source. Various other naturally occurring processes result in the formation of radical species. Oxidative phosphorylation occurs in the mitochondrion, and the organelle responsible for the next highest burden of ROS is the smooth endoplasmic reticulum (ER). Various oxidative processes, such as hydroxylation, dealkylation, deamination and oxidation are catalysed by mixed function oxygenases (MFOs). MFOs are haeme-containing proteins that catalyse the addition of the oxygen atom to various substrates using NADH/NADH as an electron donor (Voet and Voet, 1995).

A subset of the MFOs, the cytochrome P450s, is well known to those studying insecticide resistance mechanisms as a mediator of xenobiotic metabolism (Hemingway and Ranson, 2000). Cytochrome P450s are, however, a major source of ROS (Figure 4.1.1). Other oxidases are also capable of ROS production. These include xanthine oxidase, urate oxidase and

NAD(P)H oxidase, which all produce superoxide as a result of the oxidation of their substrates (Murata *et al.*, 2013, Tammariello *et al*., 2000).

Natural cellular processes are not the only sources of ROS production. Radicals can also be produced in response to an external stress. Ionising radiation, xenobiotics, and even the heat shock response have all been demonstrated to induce the production of ROS (Korsloot *et al*., 2004).

It may therefore be simple to assume that ROS are dangerous and destructive molecules. Although this is true, they do play vital biological roles, which will be discussed later. The major focus of this chapter will be the excess accumulation of radicals and the loss of homeostatic balance of ROS - known as oxidative stress.

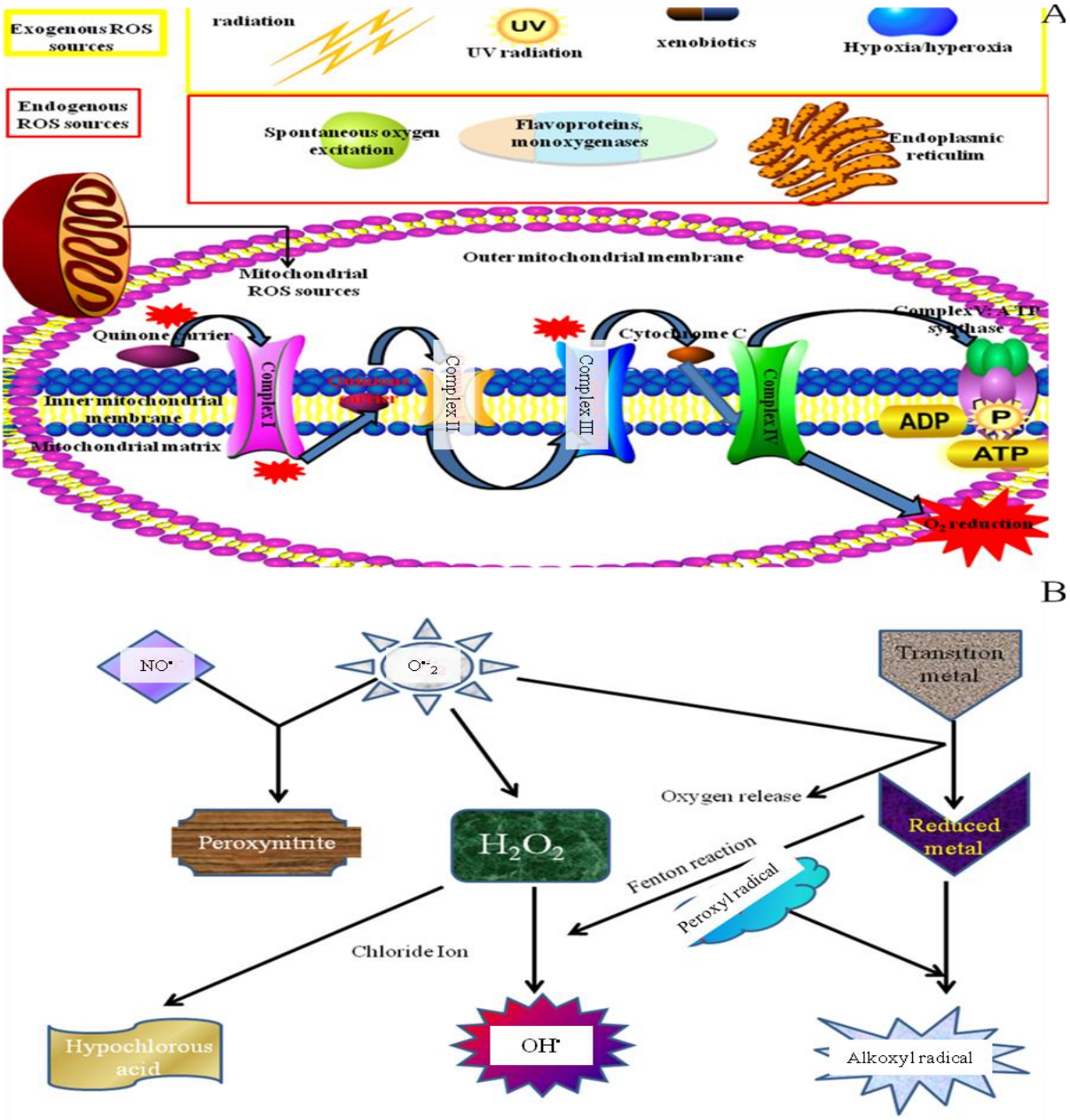


Figure 4.1 1: Cellular sources of reactive oxygen species (ROS) and the interaction of free radicals in the cell. The various sources of ROS are demonstrated in (A). External sources of ROS are highlighted in yellow, radical sources found naturally in the cell are highlighted in red. The sites of ROS production within the electron transport chain within the mitochondrion are highlighted with white blocks and red stars. The principle of radical chain reactions are highlighted in (B), demonstrating how both radical oxygen species (white blocks) and non-radical oxygen species are generated. The role of transition metals in the formation of the hydroxyl radical ($OH^{\bullet}$) via Fenton chemistry and the alkoxyl radical is also highlighted, as are the interactions of nitric oxide ($NO^{\bullet}$) and the superoxide radical ($O^{\bullet-}_2$) (Kohen and Nyska, 2002, Korsloot *et al.*, 2004, Voet and Voet, 1995).

4.1.3 Oxidative Stress

Oxidative stress is the pathological state that arises due to the disturbance of the homeostatic regulation of pro-oxidants and their antagonists, antioxidants. Oxidative stress is the result of the concentration of ROS becoming too high for the cellular environment to cope with (Halliwell, 1994). ROS are highly reactive and will interact with all biological macromolecules and are capable of causing damage.

When a radical and a non-radical react, it starts a free radical chain-reaction, which results in the formation of new radicals. This leads to the accumulation of destructive ROS which damages the major biological molecules (Betteridge, 2004). The 3 major molecules affected are lipids, proteins and DNA.

4.1.3.1 Lipids

Polyunsaturated fatty acids (PUFAs) are particularly prone to free radical attack. As these molecules are the primary constituents of cell membranes, oxidative damage to lipids is a major concern. Oxidative damage to lipids is known as lipid peroxidation, and accounts for the majority of oxidative stress mediated pathologies (Pardini, 1995). Lipid peroxidation is a three-step reaction that results in the formation of a lipid peroxide moiety which is capable of further decomposing into aldehydes, cyclic endoperoxides or isoprotans. Like the initial free radical interaction, one initiation of the lipid peroxidation process can result in a chain reaction which can subsequently lead to the peroxidation of all PUFAs in the membrane (Kohen and Nyska, 2002).

There are many deleterious consequences of lipid peroxidation. This process results in the loss of membrane fluidity and selective permeability, and membrane integrity is not only compromised, but may be lost completely. The loss of structural integrity can result in the diffusion of damaging oxidants into other cellular components. Aldehyde degradation products can cross-link proteins, causing them to aggregate. Oxidised lipid products can also interact with DNA and inhibit various enzyme activities (Betteridge, 2004).

Lipid peroxidation can therefore be considered the precursor to all other macromolecular oxidative damage. The lipid peroxide breakdown product malondialdehyde is the major lipid-induced cross-linker of DNA. 4-hydroxynonenal (HNE) is the major protein inhibitor via its' reactivity with thiols. This results in the inhibition of enzymes involved in nucleic acid and protein synthesis, as well as the inhibition of respiratory enzymes (Benedetti *et al*., 1981, Benedetti *et al*., 1980).

4.1.3.2 Proteins

Proteins are the second most abundant cell membrane components, and are also subject to oxidative assault. The hydoxyl, alkoxyl and nitrogen-reactive radicals are the predominant source of protein damage. Proteins with sulfhydryl (-SH) groups are prone to attack by hydrogen peroxide (Davies, 1987).

Oxidative damage to proteins occurs in either an indirect manner, due to damage by the products of lipid peroxidation, or by direct ROS damage. Protein damage can lead to damage of specific amino acids, which render them catalytically inactive. Oxidised proteins are also prone to degradation and fragmentation. As a result enzyme activity is compromised, cellular functions are altered and oxidised proteins are also targeted for inactivation and degradation. Typical protein oxidation products are aldehydes, keto-compounds and carbonyls (Dean *et al*., 1991).

4.1.3.3 Nucleic acids

Although DNA is a more stable macromolecule than either lipids or proteins, and it is considerably well protected by its' conformation, it is not safe from oxidative attack. ROS can result in various types of DNA damage: single and double stranded breaks, base modification, purine loss, DNA-protein cross-linkage and compromise of the DNA repair system (Poulsen *et al*., 2000).

Unlike the other major macromolecules, DNA is not susceptible to damage by all ROS. Most DNA damage is attributable to the highly reactive hydoxyl radical, which results in the formation of various DNA adducts (DNA covalently bonded to a foreign object) (Wang *et al*.,

1998). Less reactive ROS, like hydrogen peroxide, do not damage DNA at physiological concentrations, but can serve as a source for other reactive intermediates. Certain transition metals, such as iron, have a high affinity for DNA and can catalyse the production of hydroxyl radicals, ensuring a repeated oxidative assault (Gutteridge and Halliwell, 1989; Kohen and Nyska, 2002).

RNA is also prone to oxidative attack. RNA is susceptible to peroxidation of their bases, as well as damage to the sugar phosphate backbone (Fridovich, 1978, Simic *et al*., 1989). Damage to nucleic acids disrupts replication, transcription and translation and is thought to underlie many processes that lead to cell death and senescence. This will be discussed in more depth later.

4.1.4 Antioxidants

From the above description, oxidative stress due to the accumulation of ROS has a variety of deleterious consequences. As oxidative products are unavoidable in biological systems, it is therefore not surprising that a defence system exists to control the accumulation of ROS. An antioxidant is "any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate" (Halliwell and Gutteridge, 1999).

To cope with oxidative stress, various biological strategies exist to initially evade the stressor. Often these defences are structural, such as the packaging of DNA which helps shield the genetic material from damage. Although the protein cytochrome c catalyses most of the oxygen reductions of the cell, the protein itself does not release radicals, and in that way protects itself from ROS damage (Sies, 1997).

When prevention is not successful, the system needs to intercept the radicals that are produced. This is where antioxidants come into play, as they deactivate destructive radicals. Antioxidants can be either enzymatic or non-enzymatic (Sies, 1997). Non-enzymatic antioxidants can be broadly categorised into lipid and water soluble compounds (Sies, 1993).

One of the most important lipid soluble antioxidants is tocopherol (vitamin E), which is capable of scavenging alkyl and peroxyl radicals (Packer, 1992).

Of the water soluble antioxidants, glutathione and ascorbic acid are the most important. These two compounds are of particular importance, as they are recycled in the cell and therefore small amounts of these compounds can scavenge large amounts of free radicals (Felton and Summers, 1995). Several non-proteinacious, low molecular weight compounds can chelate metal ions and prevent oxidative damage. Those include ATP, uric acid, citrate and lactate (Felton and Summers, 1995).

Non-enzymatic antioxidants are commonly found in metabolically active tissues such as the fat body, midgut and malphigian tubules of insects. The haemolymph of the insect is also a major producer of ROS, but does not have enzymatic defence against radicals (Toru, 1994). The digestive lumen is remarkably devoid of most enzymatic antioxidants (Felton and Summers, 1995). This highlights the importance of non-enzymatic antioxidants as the protection in the haemolymph is offered by uric acid, ascorbic acid, reduced glutathione (GSH) and trehalose, the primary carbohydrate of the insect haemolymph (Wyatt and Kale, 1957).

4.1.5 Antioxidant enzymes

As important as non-enzymatic antioxidants are, the majority of ROS intercepting and detoxification activity is performed by enzymes. Three major oxidoreductase enzyme families are involved in the detoxification of ROS; the superoxide dismutases, catalase and peroxidase.

4.1.5.1 Superoxide dismutase

Superoxide dismutase EC 1.15.1.1 (SOD) is a metalloprotein that catalyses the dismutation of the superoxide radical, one of the most abundant mitochondrially produced ROS. This dismutation reaction results in the formation of triplet oxygen (O_2) and hydrogen peroxide (Fridovich, 1995). SOD is a highly efficient enzyme that catalyses $O_2^{\bullet -}$ dismutation 10^4 times faster than it can occur spontaneously (Fridovich, 1983).

SODs are ubiquitous enzymes found in all aerobic organisms. They are categorised according to the metal ion co-factor in their active sites. In the eukaryotic cell, SOD exists in two forms. Cu/ZnSODs have both copper and zinc in their active sites and occur in the cytoplasm and outer membrane of the mitochondria. Various extracellular Cu/ZnSODs exist (Sturtz *et al.*, 2001). MnSODs, which have manganese as a co-factor, occur exclusively in the inner mitochondrial space (Landis and Tower, 2005). SODs are essential for aerobic life, as they are the first line of defence against free radicals. They can also be rapidly induced in cases of oxidative stress (Michiels *et al*., 1994).

4.1.5.2 Catalase

SOD, as the first line of defence against ROS, produces hydrogen peroxide. This molecule, despite not being a radical, is still a ROS, and needs to be further catabolised, or it will accumulate and induce oxidative damage. Enzymes involved in the dismutation of hydrogen peroxide are referred to as catalytically active enzymes (Zamocky *et al*., 2012).

The tetrameric metalloprotein catalase, EC 1.11.1.6, catalyses the dismutation of hydrogen peroxide to oxygen and water. They are abundant proteins with a high turnover rate (Percy, 1984). Three catalase families exist. Haeme catalases (KatEs) are monofunctional, while haeme catalase-peroxidases (KatGs) are bifunctional. The final class of catalases are not haeme proteins, but have manganese in their active site (MnCats) (Zamocky *et al*., 2008). Like SOD, catalases are ubiquitous enzymes found in all aerobic organisms. In eukaryotic organisms they are commonly found in peroxisomes (Michiels *et al*., 1994). In insects, catalase is important as it is the only antioxidant enzyme found in the insect gut (Felton and Summers, 1995). Catalase is particularly important to haematophagous insects, as the silencing of catalase in *An. gambiae* results in the death (Magalhaes *et al*., 2008).

4.1.5.3 Peroxidases

Peroxidases (EC 1.11.1.1x) are a large and diverse group of tetrameric proteins that catalyse the catabolism of hydrogen peroxide to water and oxygen. The selenoprotein glutathione peroxidase (GPOX) EC 1.11.1.9 is also capable of removing other lipid and organic

hydroperoxides. In the GPOX reaction hydrogen peroxide and lipid peroxides from the cytosol or mitochondria are reduced. In the process GSH is oxidised to GSSG. The oxidised glutathione (GSSG) is then reduced by the enzyme glutathione reductase (Mannervik, 1985).

In general, insects do not appear to have selenium-dependent GPOX activity (Ahmad *et al*., 1989, Ahmad *et al*., 1988). They do, however, display peroxidase activity (Ahmad and Pardini, 1988, Sheehan *et al*., 2001, Vontas *et al*., 2001). The action of glutathione peroxidase appears to be performed by GSTs with peroxidase activity. GSTs with peroxidase activity are capable of catabolising lipid and organic hydroperoxides, but not hydrogen peroxide itself (Weinhold *et al*., 1990). The ability of GPOX to catabolise hydrogen peroxide is replaced by ascorbate peroxidase, which can scavenge hydrogen peroxide and peroxyl radicals to produce water and ascorbic acid. Like GSH, ascorbate can be recycled, and therefore a small amount of ascorbate can cope with a large concentration of hydrogen peroxide (Felton and Summers, 1995).

4.1.5.3 Antioxidant proteins without enzyme activity

Certain proteins can act as antioxidants without acting as enzymes due to their interactions with metals. Prevention of the initial free radical chain reaction can be mediated by chelating metal ions. In this defence mechanism, proteins such as ferretin, transferrin and metallothionein play an important role (Felton and Summers, 1995). Ceruplasmin converts Fe^{2+} to Fe^{3+}, a form that can be stored in ferritins. Ferritins are iron transport proteins that can translocate iron to different cellular locations. Albumin and metallothioneins are copper scavengers. The sequestration of these transition metals prevents the Fenton reaction, preventing the formation of the highly reactive hydoxyl radical, thus attenuating lipid peroxidation (Valko *et al*., 2005). Figure 4.1.2 summarises the cellular response to free radicals.

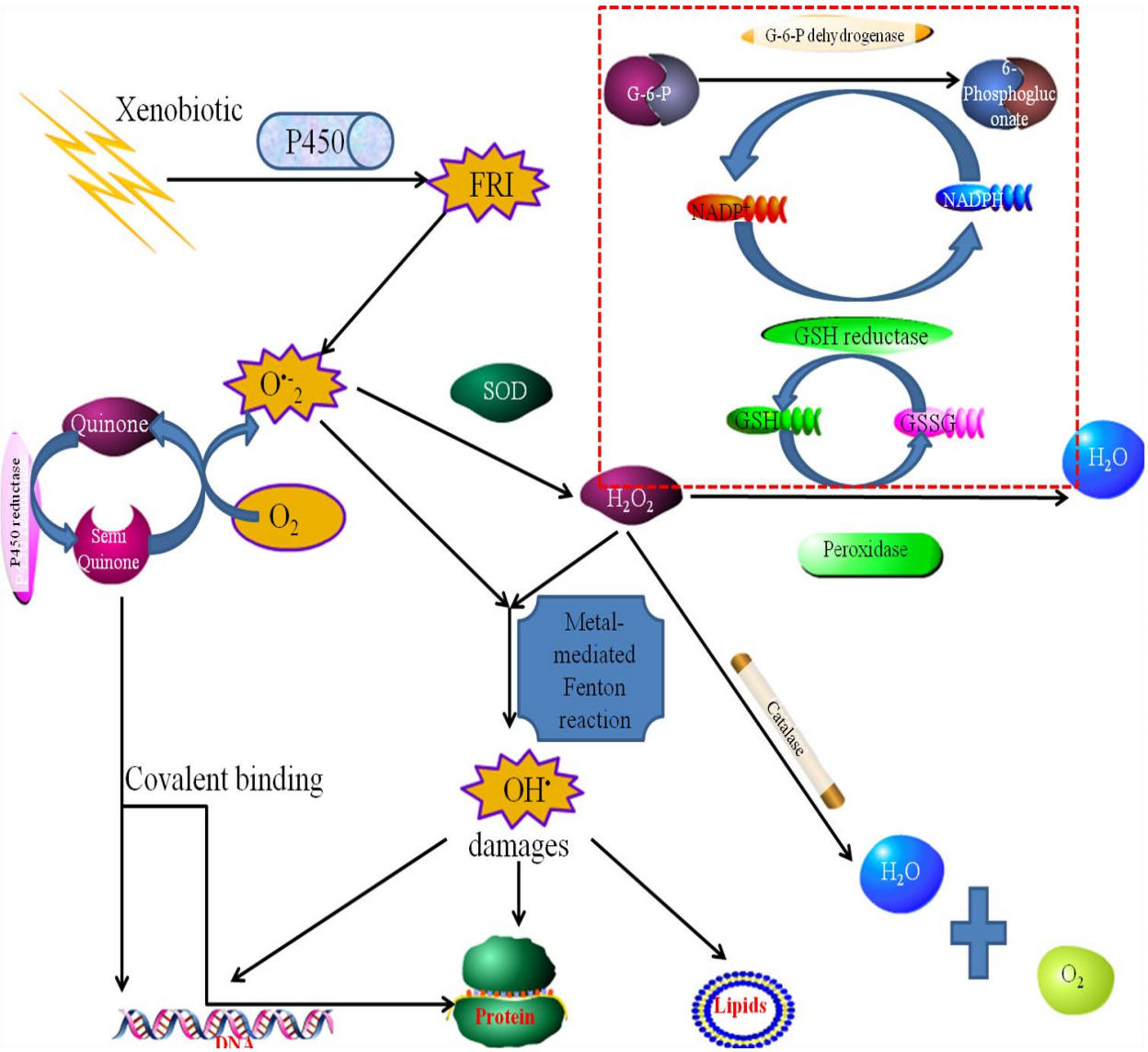


Figure 4.1.2: Defence against free radicals in the cell. Free radical intermediates (FRI), denoted by yellow multipoint stars, are generated by both the presence of xenobiotics and by the cytochrome P450s that detoxify them, as well as by the reduction of quinone electron carriers. The superoxide radical ($O_2^{\bullet -}$) is converted to hydrogen peroxide (H_20_2) by superoxide dismutases (SOD). H_20_2 is reduced to water by either catalase or glutathione peroxidase activity. The glutathione peroxidase cycling system (highlighted by the red block) requires the recycling of oxidised glutathione (GSSG) back to reduced glutathione (GSH) using the enzyme glutathione reductase. This enzyme requires the recycling of $NADP^+$ and NADH. H_20_2 that is not detoxified can be converted to the highly reactive hydroxyl radical (OH•), which is capable of damaging all three the major biological macromolecules- DNA, protein and lipids. Radicals formed by quinone redox reactions target DNA and protein (Imlay, 2003, Kohen and Nyska, 2002).

4.1.3.5 Repair systems and the antioxidant defence mechanism

Prevention of ROS formation, followed by interception of any radicals formed is the mainstay of oxidative stress defence. Neither of these systems is completely efficient, and therefore the products of oxidative damage are continuously produced at low yields, even in systems not undergoing chronic oxidative stress. As such, these products can accumulate and are capable of inducing damage. This is the most common source of DNA damage (Sies, 1997).

4.1.5 The biological roles of ROS – the redox paradox

The deleterious effect of accumulated ROS due to an imbalance in the homeostasis of these molecules has been outlined. Yet, why does the homeostasis need to exist? Why does the cellular environment not seek to completely eliminate all ROS? This is because various ROS, particularly the less reactive species such as hydrogen peroxide, also play a vital role in various cellular processes. This is known as the "redox paradox" (Goldstein *et al*., 2005). Transient bursts of ROS are triggered in response to various cellular messengers, such as cytokines, growth factors, hormones, and the transient response to the initial ligand (Goldstein *et al*., 2005).

This makes various ROS important second messengers which enhance the efficacy of various signalling molecules. Hydrogen peroxide, for example, can amplify the signalling of both insulin (May and de Haën, 1979) and transforming growth factor β (Ohba *et al*., 1994). The importance of these endogenous signals to insects will be discussed in Chapter 5. ROS themselves can therefore can also act as messengers during cell growth and differentiation (Sauer *et al*., 2001) and can modulate signalling components such as protein phosphotases, protein kinases and transcription factors (Sharma *et al*., 2012). Nitric oxide is a reactive radical gas which can cross cell membranes easily. At low levels, nitric oxide can act as a neurotransmitter (Moncada and Higgs, 1993) and can mediate immunity (Adams *et al*., 1990). Three important biological effects of ROS will be examined in slightly more detail: cell death, ageing and immunity.

4.1.6 Cell death and redox state

Cell death can occur by one of two defined pathways. Necrosis is a passive process that occurs in response to severe stress conditions. Necrosis is characterised by the depletion of ATP, followed by cellular swelling and rupture, resulting in the spilling of ruptured content into the intracellular environment (Gores *et al*., 1990). Apoptosis, or programmed cell death, is required not only during pathological states for the removal of damaged cells, but also during growth for cell replacement and tissue remodelling (Delong, 1998). In contrast to necrosis, apoptosis is characterised by cell shrinkage, chromatin condensation and DNA fragmentation. It results in the formation of apoptosis bodies (Chandra *et al*., 2000).

Intracellular radical generation is seen as a conserved apoptotic event, but some free radicals can also attenuate apoptosis. Redox metabolism therefore plays a regulatory role in cell survival (Chandra *et al*., 2000). The nature of the effect ROS appears to depend largely on the concentration of the molecules in question. Low doses of ROS induce cell proliferation as they are mitogenic. Intermediate doses results in either temporary growth arrest or replicative senescence. High doses of ROS result in cell death, either due to apoptosis or necrosis (Martindale and Holbrook, 2002).

ROS mediation of cell survival appears to be largely mediated by their capacity to effect gene expression, usually by affecting transcription factor activities. The effect of stress activated protein kinases (SAPKs) such as c-Jun N-terminal kinases (JNK) and p38 kinases is modulated either directly or indirectly by ROS. Hydrogen peroxide can directly activate SAPKs. In contrast, the antioxidant protein GST can directly bind to JNK and inhibit its' activation (Martindale and Holbrook, 2002).

The tumour suppressing protein p53 can serve as a transcription factor and regulates the expression of genes involved in growth arrest or cell death in response to genotoxic stress (Sionov and Haupt, 1999). Under basal conditions of low cell stress, p53 can activate proteins such as sestrins and TIGAR and have an antioxidant effect, resulting in the repair, protection and ultimately the survival of the cell. Under acute and persistent stress conditions proteins such as PIG3, BAX and PUM13 are activated and p53 behaves as a pro-oxidant and pushes

the cellular environment towards apoptosis (Polyak *et al.*, 1997; Martindale and Holbrook, 2002). Small shifts in cellular redox state can therefore influence the biological response of p53, and various other molecules involved with cell survival. This would affect the longevity of an organism (Vousden and Lane, 2007).

4.1.7 ROS and ageing

Theories about ageing constitute one of the most controversial subjects in biology. Medvedev (1990) summarised more than 300 theories of ageing, none of which can be described as the definitive theory of biogerontology (Medvedev, 1990). Confounding the problem is that the most popular models for ageing research, the yeast *Sacchromyces cerevisiae*, the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* and the mouse *Mus musculus*, all support different theories of ageing (Clancy and Birdsall, 2013). As *D. melanogaster* is the most closely related model to mosquitoes, a brief examination of the role of ROS in the ageing fly will be mentioned.

The free radical theory of ageing is most strongly supported by *D. melanogaster*. This theory proposes that accumulated ROS damage eventually leads to structural and functional dysregulation which ultimately leads to cellular senescence and death (Vousden and Lane, 2007). The *Drosophila* model supports this theory best, as it demonstrates all the key caveats of this theory. Four major pieces of evidence support the free radical theory; reduced mitochondrial ROS production, reduced mitochondrial activity, and consequently lowered basal metabolic rate are all associated with increased longevity in *Drosophila*. Furthermore, overexpression of Cu/ZnSOD increases lifespan (Raha and Robinson, 2000). Oxidatively damaged products also tend to accumulate with age (Landis and Tower, 2005).

The implication of the free radical theory in *Drosophila* is that ROS modulation could affect longevity in dipterans. Yet the majority of ageing studies in *Drosophila* focussed on the extrapolation of the data to humans, rather than looking at the effect of ROS on dipteran longevity. A single recent publication examined the effect of ROS on mosquito longevity. Otali *et al.* (2014) found that insecticide resistant *An. gambiae* had increased populations of mitochondrial ROS which subsequently led to reduced lifespan in these mosquitoes.

4.1.8 ROS and insect immunity

Insect immunity is a major concern for vector biologists, as it is one of the key mediators of vector competence. The involvement of ROS in anopheline immunity has been the subject of research, but contradictory evidence has been presented as to the role of ROS in immunity.

The first and most commonly held viewpoint is that ROS are essential for immunity against both the *Plasmodium* parasite and bacterial assault. The activity of Nitric Oxide synthase, an enzyme involved in the production of Nitric Oxide has been shown to be increased after *P. berghei* infection (Luckhart *et al*., 1998). Higher systemic levels of ROS were associated with increased survival after bacterial challenge. Furthermore, after a *P. berghei*-infected blood meal, catalase was not induced, as it normally is after a blood meal, resulting in higher levels of ROS after the meal. The increased oxidative burden was thought to limit *Plasmodium* infection by parasite lysis (Molina-Cruz *et al*., 2008). *Plasmodium* refractory mosquitoes were also found to be in a state of chronic oxidative stress, which was exacerbated after a blood meal. The increased levels of ROS were associated with increased immune activation resulting in heightened parasite melanisation. It was also concluded that ROS-mediated parasite damage was the underlying mechanism, although this study proposed that the damaged parasite elicited improved parasite recognition (Kumar *et al*., 2003).

The increased ROS levels in refractory mosquitoes, is not only a mosquito-mediated occurrence. In *An. gambiae*, ROS generated by the endogenous gut bacterium *Enterobacter* Ag1 acts independently to interfere with parasite development and this results in an anti-*Plasmodium* effect (Cirimotich *et al*., 2011). The role of ROS in immunity is not limited to anophelines and *Plasmodium*. Radical involvement has also been implicated in the immunity of the sandfly *Lutzomyia longipalpis* to the protozoan parasite *Leishmania mexicana* and the bacterium *Serratia marcescens* (Diaz-Albiter *et al*., 2012).

Although this theory has support, it is not unopposed. Bahia *et al.* (2013) reported a contradictory result in *An. aquasalis*' response to *P. vivax* infection. They reported no difference in systemic oxidative stress defence between infected and uninfected mosquitoes. In the midgut catalase activity was found to be reduced in response to *P. vivax* infection. After

parasite-induced suppression of catalase, parasite prevalence was found to be increased, while gut bacterial load was found to be decreased (Bahia *et al*., 2013). This is in contrast to the previously mentioned studies that found increased levels of ROS to be protective against parasites. Although this result is contradictory, it may reflect species-specific effects of ROS in both parasites and mosquito species. Most of the studies examining the effect of ROS on *Plasmodium* focussed on *P. berghei* as a model. A study examining the silencing of the oxidative stress genes Oxidation resistance 1(OXR1)Glutathione S-tranferase T1 (GSTT1) and Glutathione S-tranferase T2 (GSTT2) in the *Plasmodium* susceptible *An. gambiae* strain G3 had little effect on parasite infection (Jaramillo-Gutierrez *et al*., 2009). More work is therefore required regarding the species-specific role of ROS in immunity.

4.1.9 ROS and insecticide resistance

As alluded to earlier, despite the fact that insects, particularly flying insects, are prone to severe oxidative stress, surprisingly little investigation into oxidative stress in relation to insecticide resistance has been conducted. This is particularly true for insect vectors of human disease.

Vontas *et al.* (2001) first demonstrated that GSTs were involved in pyrethroid resistance in the brown planthopper *Nilaparvata lugens*. GSTs reduced pyrethroid toxicity by reducing the insecticide-induced oxidative stress. Although this is an important finding; before that period GSTs were not associated with pyrethroid resistance as they were not capable of direct metabolism, the impact of this often-cited finding is not appreciated. It is now the predominant theory about the role of GSTs in pyrethroid resistance and is more widely accepted than the theory that GSTs mediate pyrethroid resistance by sequestration of the insecticide. This conclusion was reached by competitive inhibition of the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) with a pyrethroid rather than direct evidence of GST-mediated pyrethroid sequestration (Kostaropoulos *et al*., 2001).

The first indication that oxidative stress defence may be an important factor in insecticide resistance was due to a study of *An. arabiensis* in Cameroon. In these pyrethroid-resistant mosquitoes, it was found that genes typically associated with pyrethroid resistance in the *An. gambiae* complex, such as CYP6P3, was downregulated in the gravid mosquitoes, and that

antioxidant genes such as Superoxide dismutase 2 (SOD2), Superoxide dismutase 3A (SOD3A) and Thioredoxin Peroxidase 4 (TPX4) (Muller *et al*., 2008) were upregulated. The authors suggest that a shift towards lowered oxidative stress was associated with pyrethroid resistance (Muller *et al*., 2008). Further microarray studies demonstrated the constitutive upregulation of oxidative stress genes in *An. funestus* (Christian *et al*., 2011) and in *An. arabiensis* with multiple resistance, including pyrethroid resistance (Nardini *et al*., 2012). Furthermore, very little attention was paid to the fact that the first colonized strain of *An. funestus*, which had basal levels of pyrethroid resistance, had not only elevated cytochrome P450 activity, but also elevated GST activity (Brooke *et al*., 2001).

Aims

Redox state plays an important role in biology, and yet remains a relatively unexplored field in anophelines. The aim of this study was therefore twofold. The first was to elucidate the role of oxidative stress in insecticide resistance, the second was to determine whether ROS plays a role in longevity, a crucial fitness determinant and component of vector competence.

4.2 Materials and Methods

4.2.1 Materials

The following strains were used (their rearing conditions and resistance mechanisms are described in chapter 2):

Laboratory Strains

Anopheles arabiensis

- **SENN**: An insecticide susceptible strain, colonised from Sennar, Sudan.
- **SENN DDT**: A DDT-selected strain of SENN that exhibits organochlorine, pyrethroid, and organophosphate resistance. Resistance is based on *kdr* as well as upregulated cytochrome P450, GST and esterase activity.

Anopheles funestus

- **FANG**: An insecticide susceptible strain colonised from southern Angola.

- **FUMOZ**: A strain exhibiting pyrethroid resistance colonised from Mozambique. No insecticide selection was performed on this strain

- **FUMOZ R**: A strain selected from FUMOZ using permethrin. It displays resistance to permethrin, deltamethrin and lambda-cyhalothrin.

Anopheles gambiae

- **SUA**: An insecticide susceptible strain from Suakoke, Liberia. This strain is *An. coluzzii* strain, but was used as a susceptible comparison for studies involving *An. gambiae* from the field.

Field strains

- **ZamG**: F1 progeny from wild-caught *An. gambiae* adults obtained from Nchlenge, Zambia, collected in January 2014. Parents displayed resistance to DDT and deltamethrin.

- **Clade I**: F1 progeny of wild-caught *An. funestus* females caught in Nchelenge, Zambia in January 2014. Parents were identified as *An. funestus* Clade I as described by Choi *et al.*, (2013). Parents displayed resistance to deltamethrin and bendiocarb.

- **Clade II**: F1 progeny of wild-caught *An. funestus* females caught in Nchelenge, Zambia in January 2014. Parents were identified as *An. funestus* Clade II as described by Choi *et al.*, (2013). Parents displayed resistance to deltamethrin and bendiocarb.

4.2.2 The oxidative burden induced by insecticides

This experiment was designed to determine whether all classes of insecticides induce oxidative stress, and if they induce similar levels of oxidative damage.

3-day-old SENN DDT females that had never taken a blood meal were exposed to standard WHO bioassays. 25 adults were exposed per tube, and 5 tubes were used per replicate. This

was repeated twice (a total of 3 replicates) with adults that emerged from separate egg batches. 3-day-old female SENN DDT adults were exposed to either DDT, permethrin, deltamethrin or malathion for 1 hour. Females that survived exposure were collected 24 hours post-exposure and immediately killed by freezing at -70^{o}C.

Protein carbonyl content, a common marker of oxidative stress, was determined according the method of Reznick and Packer (1994), and the procedure is detailed in chapter 1. The carbonyl content was compared with samples drawn from an unexposed control of the same age.

4.2.3 The effect of insecticide exposure on longevity

Although resistant mosquitoes survive doses of insecticide that would kill susceptible mosquitoes, is there a cost to this survival? This experiment was designed to determine whether insecticide exposure significantly reduced longevity of the survivors. As with experiment 4.2.2, 3-day-old female SENN DDT adults were exposed to either DDT, permethrin, deltamethrin or malathion for 1 hour. The same experimental procedure was performed as for experiment 4.2.2, but the surviving females were placed in cages and provided with a 10% sucrose solution. 30 females per cage were assessed for longevity. They were not allowed to mate or have blood for the duration of their lives. Each experiment was replicated three times, with the exception of malathion, where due to survival numbers, the experiment was repeated twice. Longevity was monitored daily with cadavers removed on a daily basis until all individuals were dead. Mortality rates were determined using the Kaplan-Meier estimator, with the Log-rank test used to determine significance as none of the data were censored.

4.2.4 The phenotypic effect of oxidative stress on resistant and susceptible mosquitoes

The aim of the following experiments was to determine whether insecticide resistant and susceptible adults have differing capacities for coping with oxidative stress, and whether a change is stress resistance has phenotypic consequences.

4.2.4.1 The effect of copper on longevity

Copper sulphate is commonly used in agriculture to increase the toxicity of pesticides (Ahmed and Shoka, 1994, Mesbah *et al*., 2002). One of the major reasons for the toxicity of copper sulphate is that it induces oxidative stress (Gupta *et al*., 2005). In this experiment, copper sulphate was used as an oxidative stressor, to determine the effect of the accumulation of ROS on the longevity of insecticide resistant and susceptible *An. arabiensis*. Females are used in this experiment as they have a higher capacity for oxidative stress defence due to their haematophagous nature. They therefore represent a good initial starting point in an examination of oxidative stress phenotype.

10mM Copper sulphate dissolved in 10% sucrose solution was supplied to SENN and SENN DDT adults. 50 newly-emerged, unmated females were used per cage, and were not allowed access to blood. Adults from each strain were kept separately. The copper solution was changed daily, and, as in experiment 4.2.3, longevity was monitored daily and cadavers were removed on a daily basis. 50 females of each strain that were allowed only 10% sucrose solution constituted the control. As with the experimental samples, the control females were newly emerged, unmated and not allowed access to blood throughout their lives. The Kaplan-Meier estimator was used to determine longevity, and significance was determined using the log-rank test, as none of the data were censored. This experiment was replicated three times.

4.2.4.2 Lethal doses of hydrogen peroxide on insecticide resistant and susceptible anopheline populations

Hydrogen peroxide is a relatively stable oxygen radical that can diffuse through cellular compartments and can cause oxidative damage (Sies, 1997). The capacity to cope with hydrogen peroxide is commonly used as a measure of oxidative stress in *Drosophila melanogaster*. Hydrogen peroxide is administered to the flies either in a food medium or by direct injection (Kaneuchi *et al*., 2003). As direct injection of hydrogen peroxide is a more viable for brachycerous flies such as *Drosophila*, it was decided to offer hydrogen peroxide as an oxidative challenge to the mosquito via sucrose solution.

In the previous experiments, the aim was to determine whether or not insecticide resistant and susceptible adults respond differently to oxidative challenge. In this experiment, the aim was to determine whether there is variation in response depending on insecticide resistance mechanisms. The *An. arabiensis* mosquitoes used in this study mediate resistance by both target site resistance and metabolic detoxification, but the *An. funestus* strains mediate resistance by metabolic detoxification only.

A dose-gradient of hydrogen peroxide was established by dissolving appropriate amounts of hydrogen peroxide in 10% sucrose to obtain final concentrations of 0%, 5%, 10%, 15%, 20% and 25%. Pure hydrogen peroxide (30%) was not used as mosquitoes would not consume it. A container of each concentration was placed in separate cages. 75 3-day-old, unmated, non-blood fed adults per gender that had previously fed on 10% sucrose were starved for 12 hours prior to being introduced into the cages. Males and females were introduced into separate cages. The mortality after 24 hours was recorded and this was used to calculate mortality using a log-probit analysis. A time of 24 hours was used as all individuals were dead after 48 hours. This procedure was performed 3 times. The *An. arabiensis* strains SENN and SENN DDT were used, as were the *An. funestus* strains FUMOZ and FUMOZ R. The fully susceptible FANG strain was not used in this experiment, as the aim was to determine the effect of resistance mechanism on oxidative stress resistance and FANG is not genetically related to the FUMOZ R strain. Thus any changes observed could be related to insecticide resistance phenotype only.

4.2.5 The biochemical basis of oxidative stress defence in insecticide resistant and susceptible anophelines

Three major enzyme systems participate in the defence against oxidative stress; the superoxide dismutases, catalase and glutathione peroxidases. When coping with respiratory-induced oxidative stress, glutathione peroxidases and catalase cope with hydrogen peroxide produced by superoxide dismutase (Halliwell, 2006). In the following experiments, the activity of catalase and glutathione peroxidase in laboratory-reared and F1 progeny of wild caught anophelines was examined.

4.2.5.1 Catalase activity

Catalase activity was determined according to the method of Sinha (1972), adapted for microplate assay and optimised for mosquitoes. The method is detailed in chapter 1.11.15.2.

5-7-day old adult males and females from the following strains were collected and killed at -70^{o}C: *An. gambiae*-SUA and ZamG; *An. funestus*-FANG, FUMOZ, FUMOZ R, Clade I and Clade II. This particular age group was used due to logistical issues with collecting the F1 adults. Single mosquitoes were homogenised in 50mM 0.1 M potassium phosphate without any protease activity, and all assays were performed within 48 hours of homogenisation. Protein was quantified by the Bradford method (Section 1.11.3.1), with Bovine Serum Albumen (BSA) as a standard curve.

Catalase activity was determined by calculating the rate of hydrogen peroxide consumption. The amount of hydrogen peroxide was quantified by a hydrogen peroxide standard curve. A total of 96 laboratory reared mosquitoes per gender per strain were assayed, while 48 F1 mosquitoes per gender were assayed due to limited availability of mosquitoes.

4.2.5.2 Glutathione peroxidase activity

The glutathione peroxidase activity for the specimens prepared in 4.2.5.1 was determined using the method of Vontas *et al.* (2001). This method is detailed in chapter 1. The specimens homogenised in 4.2.5.1 were also used for peroxidase activity determination. Glutathione peroxidase activity was determined by calculating the rate of reduced NADPH consumption. The amount of NADPH was quantified using a NADPH standard curve. For this assay a total of 96 laboratory reared mosquitoes per gender per strain were assayed, while 48 F1 mosquitoes per gender were assayed due to limited availability of mosquitoes.

4.2.6 The effect of oxidative stress enzymes on insecticide resistant phenotypes

One of the most common methods of determining whether a class of enzymes is involved in insecticide resistance is by chemically nullifying them using a chemical inhibitor. This is commonly performed on the three major detoxification enzyme systems. Piperonyl butoxide

inhibits cytochrome P450s, Triphenyl phosphate inhibits esterases, and Diethyl maleate (DEM) and S,S,S,-tributyl phosphorotrithioate (DEF) inhibits GSTS (Yu, 2008). In mosquitoes, these synergists are commonly administered by exposing the insects to filter paper impregnated with synergists prior to insecticide exposure.

Inhibiting oxidative stress enzymes in insects has been performed before. Vontas *et al.* (2001) used ethacrynic acid to inhibit GSTs with peroxidase activity. This was performed by painting the inhibitor onto brown leafhoppers. As with hydrogen peroxide, injection is not a feasible or practical option with nematocerous flies such as mosquitoes. Gupta *et al.* (2005) used the inhibitors ATZ 3-Amino-1,2,4-triazole (ATZ) and Sodium diethyldithiocarbamate (DDC) to synergise SODs and catalase in *Drosophila* respectively, and administered the inhibitors via food. Haematin is a proven GST (Ahmad *et al*.,1991; Kostaropoulos *et al*., 2001) and was used as a GST synergist. As none of these inhibitors were amenable to paper impregnation, it was decided to administer the inhibitors via sucrose, in a manner analogous to Gupta *et al.* (2005). 2mM DDC dissolved in PCR grade water, 2mM ATZ dissolved in ethanol to a final molarity of 0.017M and 20mM haematin dissolved in ammonia to a final concentration of 0.36mM was supplied via a supplemented sucrose solution to newly emerged adults.

To ensure that each synergist was not inducing death by itself, equal numbers of adults were placed in experimental and control cages. The concentration of synergist chosen was optimised as the highest soluble concentration of inhibitor that would not induce a significant increase in mortality compared to sugar-fed controls over the 3 days they were exposed to the inhibitor. The adults were allowed to feed on the inhibitor for 3 days to allow the mosquitoes to reach standard exposure age, and to ensure that all the individuals in the cage had consumed the inhibitor. This was assumed as that any adult that had not consumed any sucrose solution would not have survived the 3 days. The treatment was performed on males and females of the insecticide resistant SENN DDT and FUMOZ R strains. The dietary treatment was replicated 5 times, each time with a group of adults that emerged from a different egg batch.

After 3 days, the individuals were exposed to insecticide by standard WHO bioassay. 25 adults, separated by gender and strain, were exposed for an hour and mortality was scored 24 hours later. Two controls were put into place. The first control was sugar-fed adults that were

not exposed to any insecticides. This constituted an environmental control. A further synergist-mortality control was instituted by using synergist-treated individuals as unexposed controls. The results of the exposure were only analysed if both controls had a mortality range under 5%. The numbers of mosquitoes that were exposed for the synergist assays are given in table 4.2.1.

Table 4.2.1: Sample sizes (n) of *Anopheles arabiensis* (SENN DDT) and *An. funestus* (FUMOZ R) mosquitoes used for synergist bioassays by gender.

Synergist/Insecticide	SENN DDT		FUMOZ R	
	♀	♂	♀	♂
ATZ: DDT	1150	1159		
Deltamethrin	1375	1371	1100	1125
Permethrin	1370	1378	1117	1115
Malathion	1154	1211		
DDC: DDT	1279	1272		
Deltamethrin	1199	1210	1075	1083
Permethrin	1315	1329	1092	1089
Malathion	1290	1300		
Haematin: DDT	1410	1390		
Deltamethrin	1290	1295	997	990
Permethrin	1350	1362	989	997
Malathion	1310	1325		

4.3 Results

4.3.1 Insecticide induced oxidative stress

The resistant laboratory *An. arabiensis* strain SENN DDT displayed resistance to multiple insecticides and insecticide classes. Resistance is mediated by both target site mutation (L1014F) and increased metabolic detoxification (Nardini *et al*.,2012; Oliver and Brooke, 2013) . It was therefore used as a representative strain to determine whether insecticides induce oxidative stress, and if so, to determine which classes induce the most oxidative stress.

Figure 4.3.1 shows that deltamethrin (2-sample t-test: p=0.04; t=-4.45), DDT (2-sample t-test: p= 0.01; t=-4.65), permethrin (2-sample t-test: p<0.01; t=-15.53) and malathion (2-sample t-test: p=0.01; t=-8.22.) all induced significantly higher protein carbonyl levels than unexposed control females of the same age. Protein carbonyl levels did not differ significantly between deltamethrin and DDT (2-sample t-test: p=0.67; t=0.45) and permethrin and malathion (2-sample t-test: p=0.91; t=0.12).

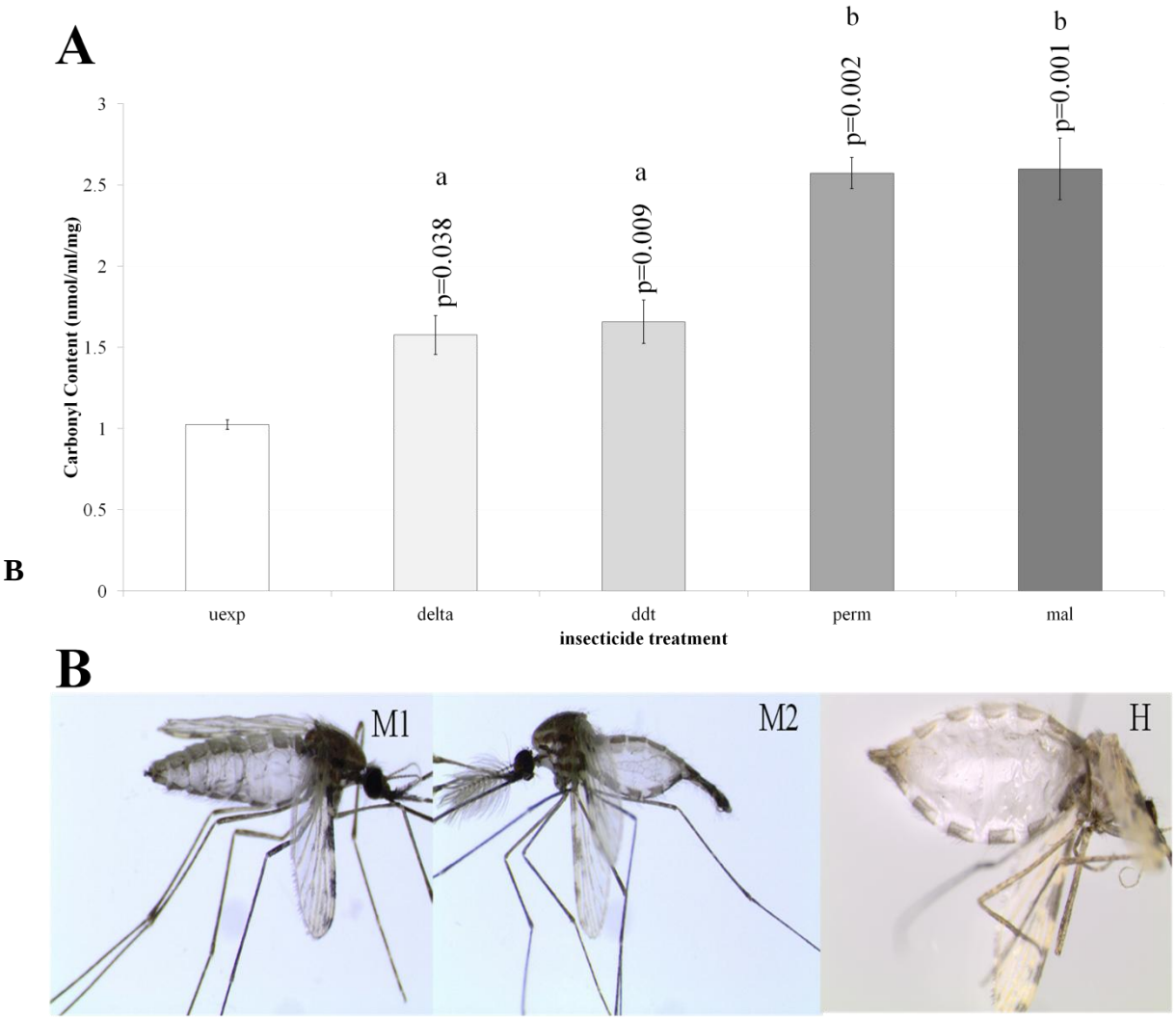


Figure 4.3.1: Oxidative stress induced by insecticides in *An. arabiensis*. The protein carbonyl content of SENN DDT females that survived exposure was quantified and compared to that of equivalent unexposed females (A). P-values indicate a significant increase in carbonyl content compared to the unexposed control. There was no significant difference between deltamethrin and DDT, nor between permethrin and malathion. (B) represents a

comparison of a common trait observed between malathion treated (M1 and M2) adults that did not survive insecticide exposure in comparison to those that succumbed to hydrogen peroxide intoxication (H). Only malathion treatment resulted in the marked abdominal expansion observed in adults that had consumed hydrogen peroxide. This expansion was observed in both sexes. Females were never mated or bloodfed, and as such, had never oviposited.

4.3.2 The effect of prior exposure to insecticides on subsequent longevity in adults

In figure 4.3.2 the effect of prior exposure to insecticide on subsequent longevity is demonstrated. Block A shows the effect of deltamethrin on longevity and Block B shows the effect of DDT on longevity. Exposure to neither of these insecticides resulted in a significant change in longevity. Block C shows the effect of permethrin exposure on longevity. In two out of three trials permethrin exposure significantly reduced the longevity of *An. arabiensis* females (Log rank test p=0.04, χ^2= 15.8; p=0.04, χ^2= 7.9). Block D shows the effect of malathion exposure on longevity. Malathion resulted in a significant reduction in longevity (Log rank test p=0.01, χ^2= 12.9; p=0.01, χ^2= 24.9).

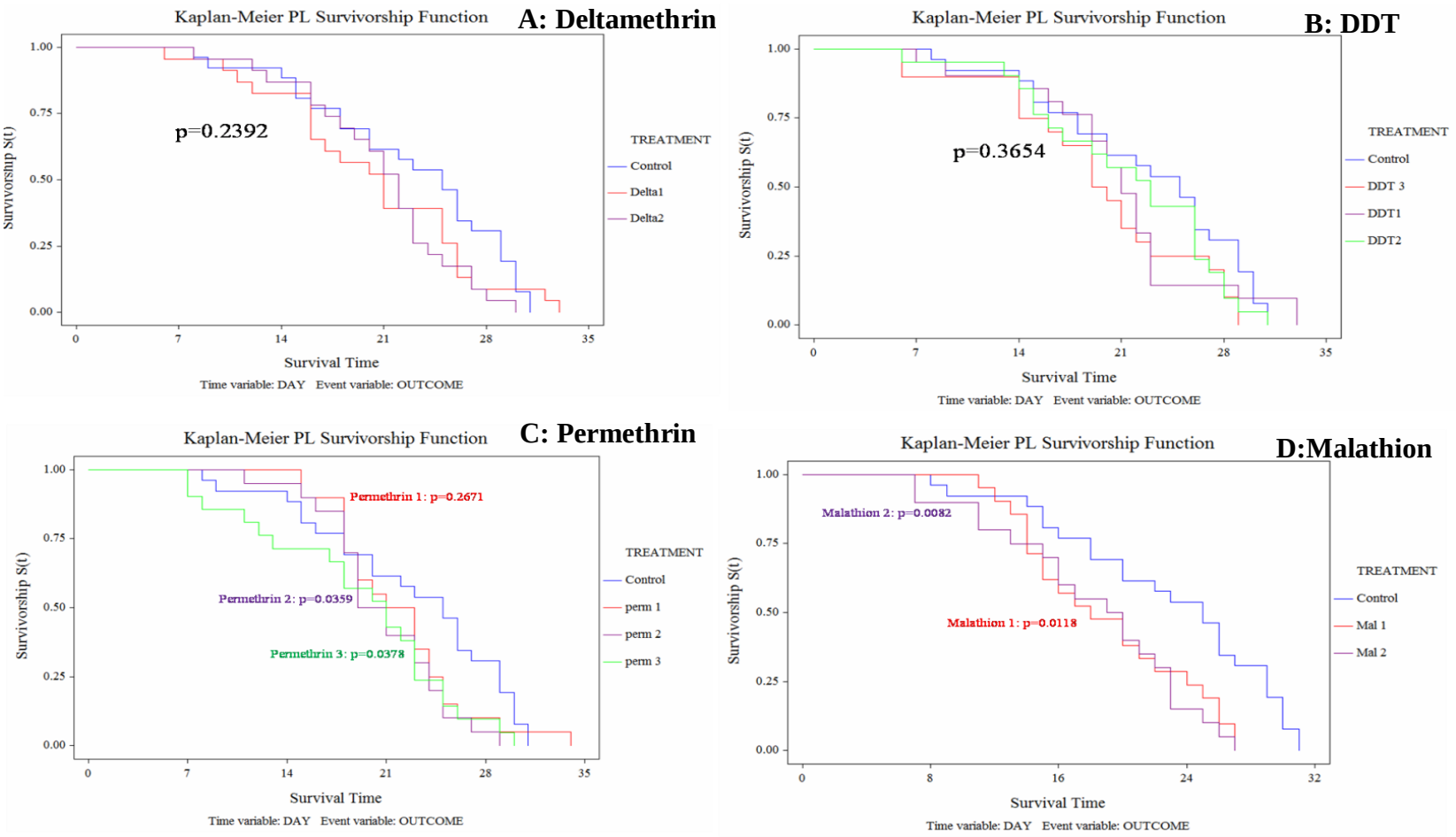


Figure 4.3.2: The effect of insecticide exposure on subsequent longevity. The longevity of *Anopheles arabiensis* SENN DDT females that survived a standard WHO bioassay was assessed. Deltamethrin (A) and DDT (B) exposures did not result in a significant change in longevity. Two out of three permethrin replicates had significantly reduced longevity (C), while malathion exposure resulted in a significant decrease in longevity with every replicate (D).

4.3.3 The phenotypic capacity of coping with oxidative stress

The previous results (figures 4.3.1 and 4.3.2) demonstrated that insecticides induce an oxidative burden, and that although resistant mosquitoes can survive an insecticide challenge, the xenobiotics that induce the greatest oxidative burden shortens their lifespan. The following set of experiments aimed to determine whether insecticide resistant and susceptible mosquitoes had differing capacities for coping with oxidative stress.

4.3.3.1 The effect of the oxidative stressor copper sulphate on longevity

Copper sulphate is an additive often used in agriculture to enhance insecticide toxicity (Kungolos *et al*., 2009) by enhancing oxidative burden. It is also used as a common metabolic stressor in the laboratory (Gupta *et al*., 2005).

Figure 4.3.3 shows that under unstressed conditions the insecticide susceptible SENN strain lived significantly longer than their resistant counterparts, SENN DDT (Log rank test $p<0.01$, $\chi^2=9.66$). Treatment with copper sulphate significantly reduced the longevity of both strains (Log rank test $p<0.01$, $\chi^2=75.31$). When comparing the longevity of the two copper treated strains, the resistant strain lived significantly longer than the susceptible strain (Log rank test $p<0.01$, $\chi^2=10.95$).

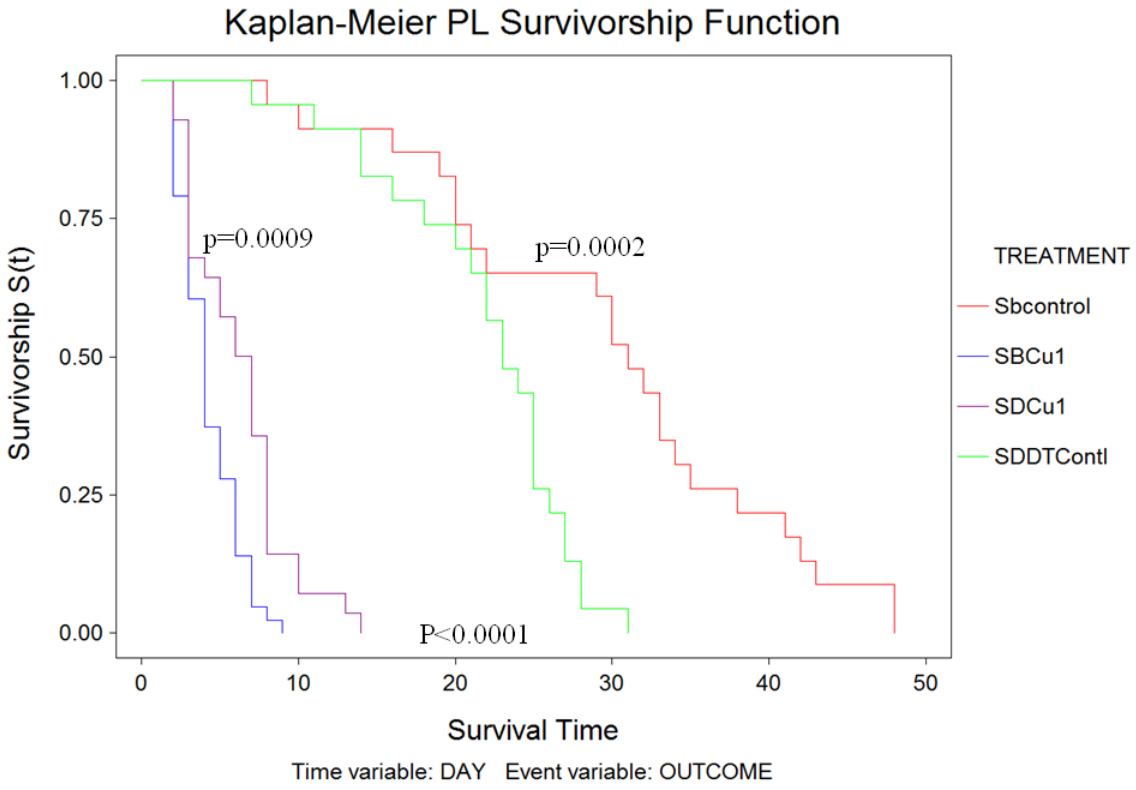


Figure 4.3.3: The effect of copper sulphate treatment on the longevity of *An. arabiensis*. The longevity of *An. arabiensis* which had been given a dietary source of copper sulphate was assessed. Under unstressed conditions SENN (Sbcontrol) lived significantly longer than SENN DDT (SDDTContl) (p=0.0002). Although both strains showed significantly shorter longevities due to copper treatment, copper sulphate treatment significantly shortened the longevity of SENN (SBCu1) in comparison to SENN DDT (SDCu1)(p=0.0009).

4.3.3.2 Lethal doses of the stressor hydrogen peroxide

The previous experiment suggests that insecticide resistant females have a greater capacity for coping with oxidative stress than their susceptible counterparts. This raises two further questions. Firstly, does this observation hold true for males as well? Secondly, does the insecticide resistance mechanism affect the resistant mosquito's capacity for coping with oxidative stress?

To answer these questions, two different species of *Anopheles* were used. The SENN DDT strain, as mentioned before, mediates resistance by multiple mechanisms, while FUMOZ R mediates resistance by metabolic detoxification only, as no target site mutations have been found in this strain (Okoye *et al*., 2008). Resistance in this strain is primarily mediated by over expression of Cyp6p13/Cyp6p9b (Amenya *et al*., 2008, Wondji *et al*., 2009).

Figure 4.3.4 summarises the LD_{15} values for laboratory strains of *An. arabiensis* and *An. funestus* strains. LD_{15} values were calculated because LD_{50} values could not be accurately calculated for the *An. funestus* strains, which were remarkably resistant to hydrogen peroxide.

For *An. arabiensis,* the LD_{15} values of males and females of the susceptible SENN strain were compared to that of the resistant SENN DDT strain. There was no significant difference in LD_{15} between SENN males and females ($p=0.22$, $t=-1.47$). In contrast, female SENN DDT had significantly higher LD_{15} values than their male counterparts (2 sample t-test: $p<0.01$; $t=-6.43$). SENN DDT males did not differ significantly from SENN males and females (1-way ANOVA; $p=0.38$;$F=0.82$).

Similar results were found in the *An. funestus* strains. FUMOZ R was selected from the FUMOZ strain, which although not fully susceptible, is a genetically related strain to FUMOZ R. In the FUMOZ strain there was no significant difference in the LD_{15}s of the males and the females (2 sample t-test: $p=0.25$, $t=-1.51$). In the FUMOZ R strain, females had a significantly higher LD_{15} than FUMOZ females (2 sample t-test: $p<0.01$; $t= 7.92$). Similarly, FUMOZ R males had a significantly higher LD_{15} than FUMOZ males (2 sample t-test: $p<0.01$; $t= 23.79$).

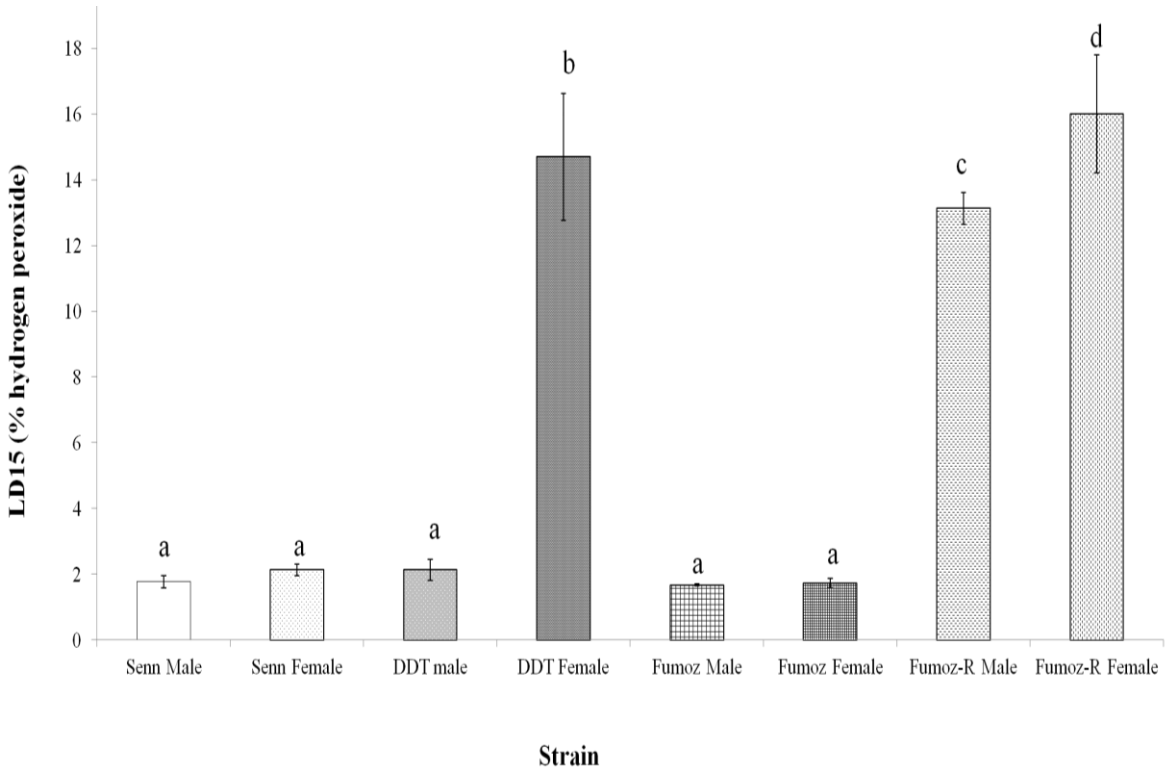


Figure 4.3.4: Lethal doses of hydrogen peroxide on two species of *Anopheles* mosquito. In *An. arabiensis*, SENN DDT females had a significantly higher LD15 than males and compared to their susceptible SENN counterparts. FUMOZ R males and females had significantly higher LD_{15}s than their equivalent FUMOZ counterparts. Significant differences are indicated by varying letters.

4.3.4 The biological basis for oxidative stress resistance

The previous experiments have demonstrated that insecticide resistant mosquitoes have an increased capacity for defence against oxidative stress, and that species-specific gender differences related to insecticide resistance mechanisms exist. The following experiments examined the activity of enzymes involved in oxidative stress. The catalase and glutathione peroxidase activity of laboratory reared *An. funestus* were and compared to the F_1 progeny of wild caught *An. funestus*. As they were available, the enzyme activities of wild caught *An. gambiae* were compared to that of a susceptible laboratory strain. The glutathione peroxidase and catalase activity of *An. arabiensis* was examined under a different set of experiments, detailed in Chapter 5.

In terms of catalase activity (figure 4.3.5A/C), FUMOZ R had higher levels of enzyme activity compared to the susceptible FANG strain for both males (2 sample t-test: $p<0.01$; $t=-1.68$) and females (2 sample t-test: $p<0.01$; $t=-3.46$). FUMOZ females had significantly higher levels of enzyme activity than FANG females (2 sample t-test: $p=0.01$; $t=-2.61$). Clade I males had significantly higher levels of enzyme activity than FANG males (2 sample t-test: $p<0.01$; $t=-3$) and Clade I females had significantly higher levels of catalase activity than FANG females (2 sample t-test: $p=0.03$; $t=-2.12$). Wild caught *An. gambiae* had higher levels of catalase activity than the susceptible SUA strain for both males (2 sample t-test: $p<0.01$; $t=-2.22$) and females (2 sample t-test: $p=0.04$; $t=3.09$) (Figure 4.3.5B).

Glutathione peroxidase activity (Figure 4.3.5 B) in FUMOZ R was significantly higher than FANG for males (2 sample t-test: $p=0.04$; $t=-2.15$) and females (2 sample t-test: $p<0.01$; $t= -3.56$). FUMOZ R males had significantly higher peroxidase activity than FUMOZ males (2 sample t-test: $p<0.01$; $t=-3.56$). Clade I males had significantly increased enzyme activity compared to FANG males (2 sample t-test: $p=0.03$; $t=-2.31$) and Clade II males (2 sample t-test: $p<0.01$; $t=-4.65$). Similarly, Clade I females had significantly higher enzyme activity than FANG females (2 sample t-test: $p<0. 01$; $t=4.88$). Clade II females also had significantly higher levels of enzyme activity than FANG females (2 sample t-test: $p<0.01$; $t=-5.11$). Unlike their male counterparts, there was a significant difference between the peroxidase activity of Clade I and Clade II females, with Clade I females having a significantly higher level of enzyme activity (2 sample t-test: $p<0.01$; $t=3.12$). Wild caught *An. gambiae* (Figure 4.3.5D) had significantly higher levels of peroxidase activity than SUA for both males (2 sample t-test: $p<0.01$; $t=13.38$) and females (2 sample t-test: $p<0.01$; $t=-6.46$).

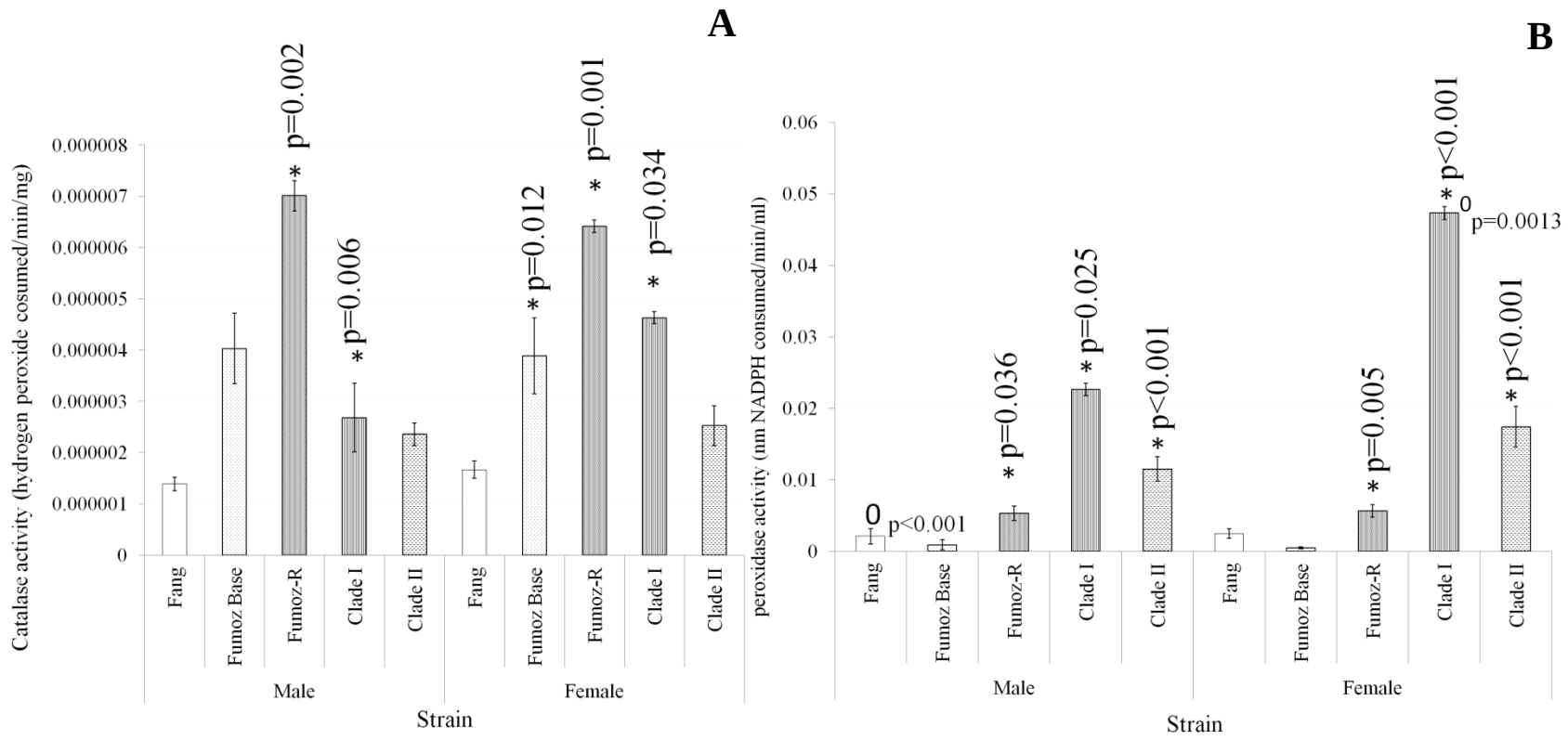


Figure 4.3.5: Comparison of ROS detoxification enzyme activity in laboratory strains and F_1 progeny of *An. funestus*. The catalase activities (A) and the glutathione peroxidase activities of *An. funestus* (B). An asterisk (*) denotes a significant difference between the strain and its' insecticide susceptible counterpart. A circle (o) indicates a significant difference between the two neighbouring strains. FUMOZ R had significantly higher levels of glutathione peroxidase activity than FUMOZ, and Clade I had significantly higher activity levels of activity than Clade II

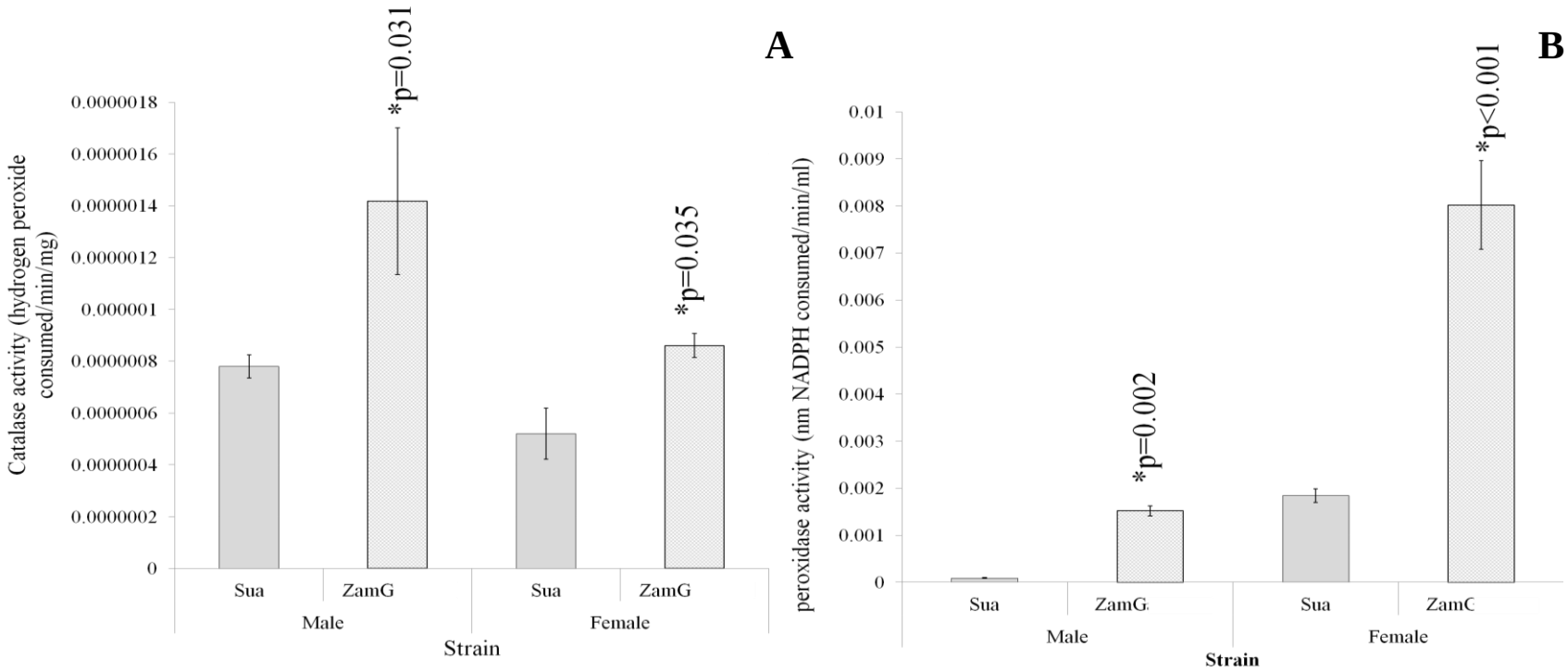


Figure 4.3.6 Comparison of ROS detoxification enzyme activity in laboratory strains and F_1 progeny. The catalase activity (A) and glutathione peroxidase activities of *An. gambiae* (B) are indicated. An asterisk (*) denotes a significant difference between the strain and its' insecticide susceptible counterpart.

4.3.5 The role of oxidative stress enzymes in the insecticide resistance phenotype

Enzyme synergism is an effective and efficient mechanism of the expression of the phenotype. At the time of writing, there were no published reports of synergism of oxidative stress enzymes in mosquitoes, and no investigation of their role in insecticide resistance.

4.3.5.1 Synergism of oxidative stress enzymes in An. *arabiensis*

The SENN DDT strain is resistant to multiple insecticides, mediated by different mechanisms (Nardini *et al*., 2012; Oliver and Brooke, 2013). Therefore the role or importance of oxidative stress enzymes may vary from that in *An. funestus*, which mediates pyrethroid resistance by a single mechanism.

Figure 4.3.6 shows the effect of oxidative stress enzyme synergism in *An. arabiensis*. In Block A, the inhibition of Cu-Zn SODs by DDC is demonstrated. A 100% mortality rate is not reached for any of the synergised mosquitoes. DDC synergism resulted in a significantly increased mortality for deltamethrin-exposed females (2 sample t-test: $p<0.01$; $t=-6.70$) and permethrin-exposed females (2 sample t-test: $p=0.04$; $t=-2.20$). For males DDC synergism resulted in a significantly increased mortality following deltamethrin exposure ($p=0.02$; $t=2.89$) as well as following permethrin exposure ($p=0.02$; $t=-3.46$) There was no significant difference in mortality for synergised and unsynergised males and females following malathion and DDT exposure.

As with DDC synergism, haematin synergism (GST inhibition) (Figure 4.3.6B) never resulted in 100% mortality. Haematin synergism was the only one of three synergists that resulted in a significant increase in mortality following exposure to all four insecticides for female SENN DDT. Synergised females showed a significant increase in insecticide-induced mortality for DDT (2 sample t-test: $p<0.01$; $t=-5.46$), deltamethrin (2 sample t-test: $p<0.01$; $t=-3.40$), permethrin (2 sample t-test: $p=0.01$; $t=-2.65$) and malathion (2 sample t-test: $p=0.03$; $t=-2.26$). There were no significant differences in mortality between synergised and unsynergised males.

Catalase synergism by ATZ (Figure 4.3.6C) resulted in the highest pyrethroid induced death. ATZ synergised females showed significantly increased deltamethrin-induced mortality (2 sample t-test: $p<0.01$; $t= -8.46$) as well as permethrin-induced mortality (2 sample t-test: $p<0.01$; $t=-2.85$). Simarlarly, ATZ synergised males showed a significant increase in deltamethrin-induced- mortality ($p<0.01$; $t=-10.35$), as well as permethrin-induced mortality ($p<0.01$; $t=-2.09$). Unlike the females, however, male ATZ-synergised mosquitoes showed a significant increase in malathion-induced mortality ($p=0.05$; $t=-3.07$).

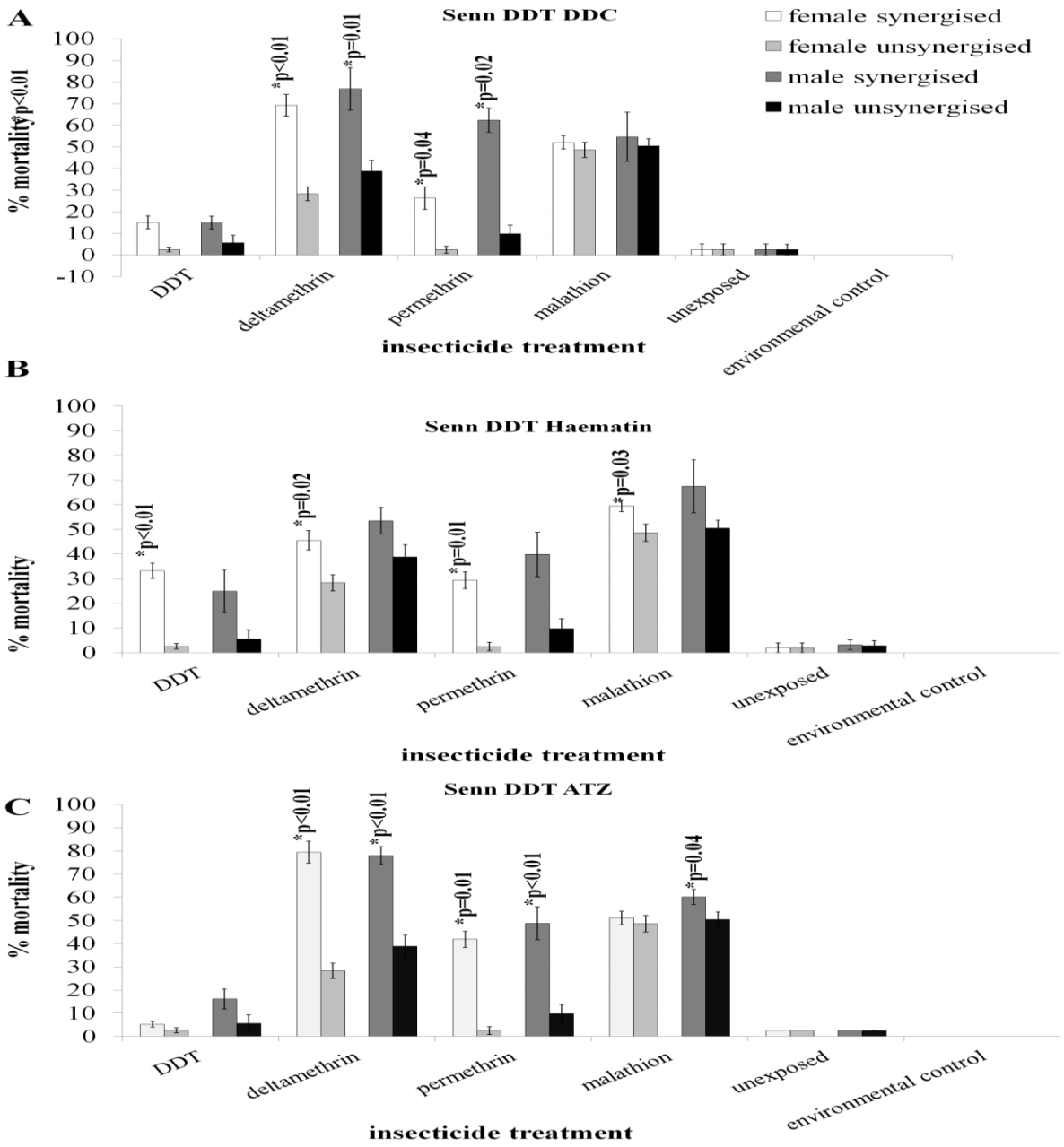


Figure 4.3.7: The effect of synergism of ROS detoxification enzymes on the insecticide resistance phenotypes of *Anopheles arabiensis* SENN DDT. SOD synergism (A) resulted in significantly increased levels of pyrethroid induced mortality in both males and females. GST synergism (B) resulted in significantly higher mortality for all insecticides for females, but did not result in any changes in the insecticide resistance phenotype of the males. Catalase synergism (C) resulted in a significant increase in pyrethroid mortality of males and females, as well as a significant increase in malathion mortality in males.

4.3.5.2 Synergism of oxidative stress enzymes in *An. funestus*

The inhibition of oxidative stress enzymes in *An. funestus* followed a similar pattern of phenotypic change as for *An. arabiensis*. This is based on the observation DDC, the SOD inhibitor, resulted in the lowest pyrethroid-induced mortality(3 fold increase in mortality), while ATZ, the catalase inhibitor resulted in the highest insecticide-induced mortality (a 7 fold increase in mortality).

The SOD inhibitor DDC (Figure 4.3.7A) caused a significant increase in permethrin-induced mortality for males (2 sample t-test: $p<0.01$; $t=-4.43$) and females (2 sample t-test: $p<0.01$; $t=-4.38$). Similarly, deltamethrin-induced mortality for synergised males was significantly increased (2 sample t-test: $p<0.01$; $t=-4.43$) as was as that of females (2 sample t-test: $p<0.01$; $t=-3.79$).

The GST inhibitor haematin (Figure 4.3.7B) induced higher mean pyrethroid-induced mortality than DDC. Haematin significantly increased permethrin-induced mortality for males (2 sample t-test: $p<0.01$; 7.44.) and females (2 sample t-test: $p<0.01$; $t=-8.66$). Likewise, synergised males showed significantly increased deltamethrin-induced mortality (2 sample t-test: $p<0.01$; $t=-6.36$) as did females (2 sample t-test: $p<0.01$; $t=-12.9$).

The catalase inhibitor ATZ (Figure 4.3.7C) resulted in the highest overall pyrethroid induced mortality, and came the closest to inducing 100% insecticide-induced mortality. As for the other three synergists, permethrin-induced mortality was increased for males (2 sample t-test: $p<0.01$; $t=-10.89$) as well as females (2 sample t-test: $p<0.01$; $t=-16.28$). Similarly, deltamethrin-induced mortality was significantly increased for synergised males (2 sample t-test: $p<0.01$; $t=-11.30$) as well as synergised females (2 sample t-test: $p<0.01$; $t=-19.68$).

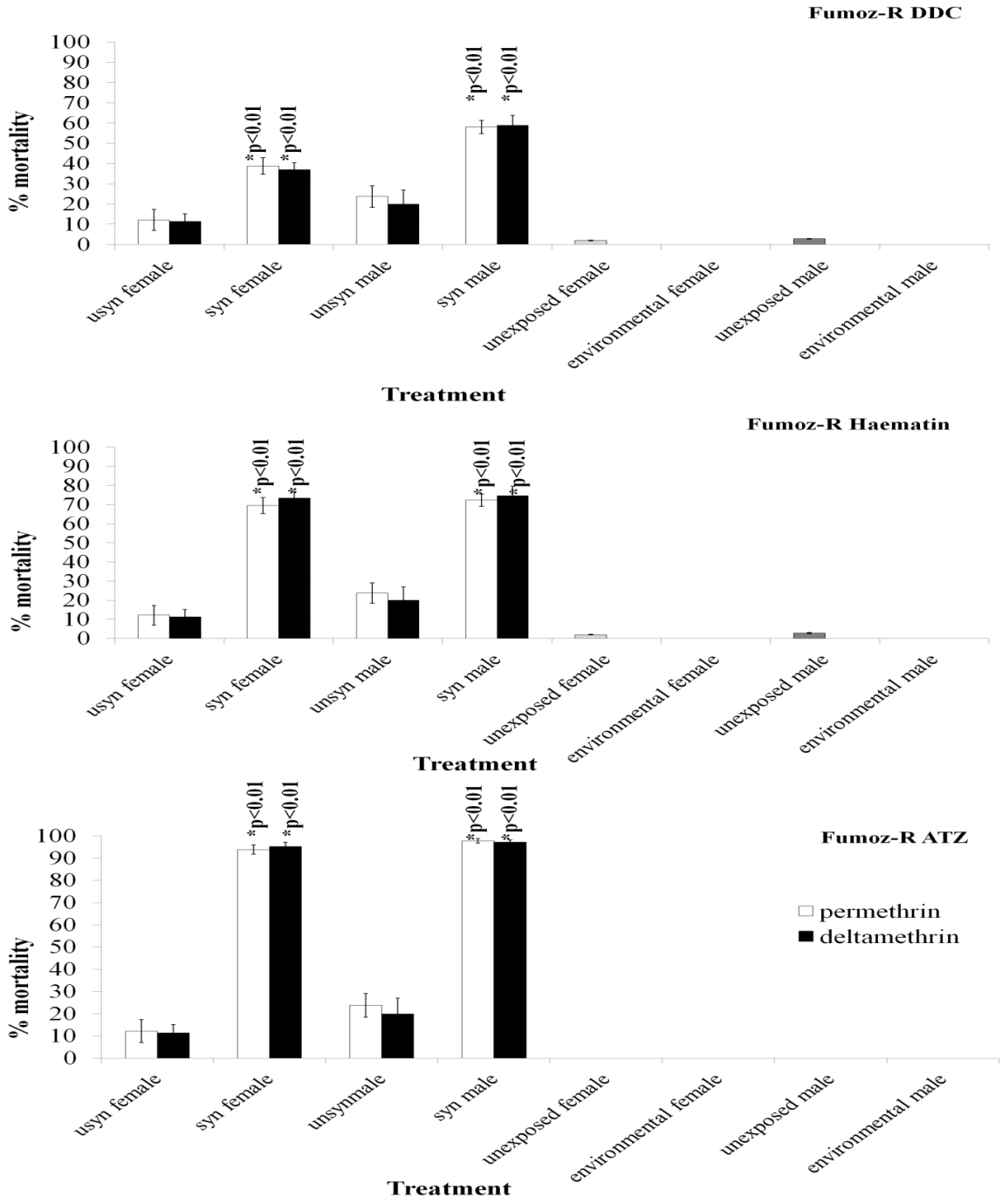


Figure 4.3.8: The effect of synergism of ROS detoxifying enzymes on the insecticide resistance phenotype of *Anopheles funestus* FUMOZ R. Synergist treatment resulted in a significant increase in permethrin and deltamethrin induced mortality in both males and females.

4.4 Discussion

The oxidative stress induced by pesticides on non-target organisms, particularly on fish and aquatic invertebrates has been a major concern for as long as it has been known that these effects exist (Slaninova *et al*., 2009). The effect of oxidative stress induced by pesticides on the target pest is of much less concern. The neurotoxic effects of the insecticides used in vector control have been the major focus of toxological studies, as the effect of oxidative assault takes longer than the neurological effects (Vontas *et al*., 2001). This study, however, shows that oxidative stress should be a major consideration in vector biology.

Although it has been known that insecticides, pyrethroids in particular, induce oxidative stress (Vontas *et al*., 2001), the differences in stress produced by various insecticides has never been quantified. Using protein carbonyl as an indicator, increasing levels of oxidative damage were induced by deltamethrin, DDT, permethrin and malathion. An unquantified observation was made that malathion was the only one of the four insecticides used for treatment that resulted in a similar occurrence to what happens when feeding adults hydrogen peroxide. Many malathion-succumbing *An. arabiensis* had ruptured abdomens (figure 4.3.1b), a common observation in individuals that succumbed to hydrogen peroxide consumption. It is possible that the abdominal rupturing is due to the oxidative stress induced by the malathion and hydrogen peroxide. It is interesting to observe that although many SENN DDT females survived permethrin treatment, this insecticide treatment resulted in the second highest induction of oxidative stress. As detailed in chapter 2, permethrin resistance in SENN DDT appears to be primarily mediated by *kdr*, while deltamethrin resistance appeared to be primarily mediated by cytochrome P450s (Nardini *et al*., 2012). This suggests that although the *kdr* mutation protects against the neurotoxic effects of permethrin by reducing the affinity of the voltage gated sodium channel to the insecticide, it does not protect against the effects of oxidative stress.

Although the degree of oxidative stress induced by insecticides may not seem important, it is of interest to note that the two insecticides that induced the most oxidative stress (permethrin, malathion) caused significant reductions in longevity. Therefore, although insecticide resistant adults are capable of surviving insecticide treatment, there is an unexpected cost in terms of reduced longevity.

The free radical theory of ageing underlies the experiment examining the effect of copper treatment on subsequent longevity. This experiment was one of a series that demonstrated that insecticide resistant mosquitoes are better capable of coping with oxidative stress. Although the longevity of treated adults was reduced compared to the equivalent sugar fed controls, copper-treated SENN DDT survived longer than insecticide susceptible SENN. Interestingly, as shown in Chapter 5, non-stressed SENN DDT adults live for a significantly shorter period than SENN adults. A similar result was found using genetically unrelated *An. gambiae*. In a recent study, resistant mosquitoes also lived for a significantly shorter time than insecticide-susceptible mosquitoes. The conclusion of the study was that resistant mosquitoes produced more mitochondrial ROS, which resulted in a reduced longevity (Otali *et al*., 2014).

In the present study of oxidative stress in anophelines, mitochondrial ROS were not directly quantified. In this chapter it was demonstrated that resistant mosquitoes cope better with oxidative stress, and in Chapter 5 it is demonstrated that SENN DDT has lower levels of oxidative damage markers than their insecticide susceptible counterparts, SENN. Otali *et al.* (2014) therefore present a model that is consistent with the free radical theory of ageing for *An. gambiae*, while the present study does not present such a simple model. The first explanation for this may be that the differences between the two studies are due to species differences. Although *An. gambiae* and *An. arabiensis* are sibling species, they vary in physiology and metabolism (Huestis *et al*., 2011). Secondly, an explanation for the reduced longevity in resistant mosquitoes based on the results presented here relates to metabolism. Resistant SENN DDT have constitutively high activity levels of the three major detoxification enzyme classes, (see chapter 5), as well as constitutively higher levels of ROS detoxification enzymes, and are more capable of

inducing peroxidase activity to cope with oxidative stress. Therefore, SENN DDT adults are generally more metabolically active and the depletion of metabolic stores may account for their reduced longevity. In aerobic organisms, increased metabolic activity, such as the increased detoxification activity, can result in the increased production of ROS (Blagojevic *et al*., 2011) An increased capacity of insecticide resistant mosquitoes to cope with oxidative stress may also be partially driven by the need to cope with additional ROS produced by the more metabolically active insecticide resistant individuals. Furthermore, in both the present study and the Otali study, the resistant strains were homozygous for the *kdr* mutation. The *kdr* mutation has been reported to be associated with several fitness costs. In *Aedes*, it has been shown to increase larval development and reduce fertility (Brito *et al*., 2013). Crucially, it has also been demonstrated to reduce longevity in adult female *Aedes aegypti* (Martins *et al*., 2012). The presence of *kdr* needs to be considered in conjunction with the oxidative stress data. Both the present study and the Otali study needs to be confirmed in wild populations.

Although the role of ROS in longevity is speculative, this study does conclusively demonstrate that defence against reactive species plays a crucial role in insecticide resistance, particularly pyrethroid resistance. An elevated capacity to cope with oxidative stressants as well as elevated oxidative stress enzymes was demonstrated in both *An. arabiensis* and *An. funestus*. The significance of these findings needs to be dissected.

The capacity to cope with the ROS hydrogen peroxide is a measure of oxidative stress defence, and indirectly, of catalase and glutathione peroxidase activity. The capacity of different species to cope with hydrogen peroxide gives some interesting insights into the possible evolution of increased oxidative stress defence in insecticide resistant mosquitoes.

The *An. arabiensis* strain SENN DDT mediates pyrethroid resistance by both target site and metabolic mechanisms. In this strain only SENN DDT females have an elevated capacity of coping with hydrogen peroxide. It is not unusual for female mosquitoes to have an increased capacity of coping with oxidative stress compared to males as they have to bear an additional oxidative burden- the blood meal. The higher basal levels of

oxidative stress defence in SENN DDT females suggests that the increase in oxidative stress defence against a blood meal in this strain will be exponentially higher in insecticide resistant mosquitoes than in insecticide susceptible mosquitoes. Therefore, the two strains will respond differently to a blood meal in terms of enzyme activity. This speculation will be examined in detail in Chapter 5.

Considering the strong gender bias in *An. arabiensis*, it is striking that male *An. funestus* (FUMOZ R) have such a marked capacity for coping with hydrogen peroxide, while their male *An. arabiensis* counterparts do not. The possible reason for this may depend on the evolution of insecticide resistance. Pyrethroid resistance in *An. funestus*, particularly in this laboratory strain, is essentially monofactorial, and is due to increased transcription and activity of cytochrome P450s, Cyp6P13/Cyp6p9b in particular (Amenya *et al*., 2008, Matambo *et al*., 2010, Wondji *et al*., 2009). In this strain, although only one cytochrome P450 is implicated in resistance, it has high levels of both haeme peroxidase activity (Brooke *et al*., 2001), as well 7-ethoxycoumarin activity (Stradi, 2011). This indicates generally high cytochrome P450 activity, and this would result in high levels of ROS production. As these elevated levels of cytochrome P450 activity are present in both males and females, both sexes would have to evolve mechanisms for coping with the continuous oxidative onslaught. In contrast, the *kdr* mutation appears to be the primary mechanism of permethrin resistance in SENN- DDT (Nardini *et al*., 2012) , with metabolic resistance playing a secondary role. The *kdr* mutation is not present in FUMOZ R, and has not been described in *An. funestus* (Okoye *et al*., 2008).

A common critique of laboratory studies on mosquitoes is that it may not be representative of the wild population. The use of F1-progeny of wild Zambian mosquitoes has a two-fold purpose in this study. Firstly, it aims to examine whether the results found in the laboratory strain are mirrored in the wild. Secondly, it is one of the first studies to try to determine whether any biological differences exist between Clade I and Clade II of *An. funestus*.

The existence of two separate clades within *An. funestus s.s.* was first described in 2005 (Michel *et al*., 2005a, Michel *et al*., 2005b). Little further investigation was done on the

subject until both clades were detected in various areas of southern Africa (Choi *et al*., 2012), and both Clade I and Clade II were found to be present in Zambia (Choi *et al*., 2013). As Clade I and Clade II may represent incipient species, it would be interesting to see if any further biological differences exist between these two clades.

Firstly, it is important to note that the patterns of catalase and glutathione peroxidase activity observed in the laboratory *An. funestus* strains are similar to that of the field strains (figure 4.3.5). In fact, the glutathione peroxidase activity of the field strains exceeded that of the laboratory strains Clade 1 has a 8.4 fold increase over FUMOZ R and a 18.9 fold increase over FANG. Clade 2 an 3.1 fold increase over FUMOZ R and an 6.9 fold increase over FANG . Synergist studies of previous field collections of Zambian *An. funestus* implicated cytochrome P450s as the primary mechanism of pyrethroid resistance (Choi *et al*., 2014). A possible explanation for the high antioxidant enzyme activity in these strains, particularly Clade I is to cope with the oxidative stress induced by the heightened Cytochrome P450 activity in these strains.

Glutathione peroxidase activity appears to be particularly important, as increases in ROS detoxification enzyme activity in insecticide resistant mosquitoes are most marked in this enzyme. Furthermore, it also represents a significant biological difference between the two clades, with Clade I having significantly higher levels of glutathione peroxidase activity than Clade II in both males and females (figure 4.3.5). This represents one of the first discernible and quantifiable differences between these two potentially incipient species.

The fact that the same patterns of oxidative stress enzyme activity are observed in *An. funestus, An. gambiae* and *An. arabiensis* (See Chapter 5, figure 5.3.7 and figure 5.3.8) indicates that elevated oxidative stress enzyme activity is not species-specific, but may represent a conserved response in insecticide resistant anophelines. Yet, although the evidence of phenotypic and biochemical studies provide a compelling argument for the increase of oxidative stress in insecticide resistant adults, ultimately, it is circumstantial. It does not directly implicate the defence of oxidative stress enzymes in the insecticide resistant phenotype. For this reason, synergism of the oxidative stress defence enzymes

was performed. Synergism of detoxification enzymes is a common and useful tool to implicate enzyme systems in a phenotypic response. It is important to note the limitations of the synergists used, and state that the use of the synergists served as an initial indicator than a final result. Now that synergism has provided baseline results, it is possible to fine-tune the process of determine exactly which oxidative stress enzymes are involved by using RNA interference. This has potential to be an interesting future study.

When examining the synergist effect in *An. arabiensis*, it was clear that the synergism of oxidative stress enzymes played a significant role in pyrethroid resistance. The SOD inhibitor DDC was the worst performing synergist, inducing the smallest increase in pyrethroid-induced mortality. The catalase inhibitor ATZ (an average of 2.8 fold increment) was the best performing synergist, increasing pyrethroid-induced mortality by the highest increment. Glutathione peroxidase activity was never specifically synergised. The synergist cibacron blue specifically inhibits GSTs with peroxidase activity (Vontas *et al*., 2001). This process, however, was difficult to optimise and as it became increasingly difficult to obtain this compound, the use of this synergist was abandoned. The general GST synergist haematin was therefore used. GST synergism usually performed better than SOD synergism, but never as well as catalase synergism.

The results of enzyme synergism are quite difficult to interpret for *An. arabiensis*, due to the complexity of the insecticide resistance mechanisms involved. In general, it can be concluded that the synergism of oxidative stress enzymes increased pyrethroid-induced mortality in *An. arabiensis*. It is of interest to note that GST synergism increased insecticide-induced mortality of all four insecticides that SENN DDT females were exposed to, but did not have any effect on the insecticide induced mortality in males for any of the treatments. This may suggest that GST is more important for insecticide resistant *An. arabiensis* females than for males. The synergism experiment is easier to interpret in *An. funestus*. This is because only two pyrethroids were used in this experiment, and only metabolic resistance was present in the strain. As with *An. arabiensis*, SOD synergism had the weakest effect, catalase synergism had the strongest effect, and GST synergism had an intermediate effect on pyrethroid resistance. Catalase

synergism almost completely restored susceptibility to pyrethroids in FUMOZ R. The implications of these results are twofold.

Firstly, it implies that defence against oxidative stress is particularly important in mosquitoes where only metabolic resistance is present. This is particularly true where cytochrome P450 is the major resistance mechanism. This is most likely so that these mosquitoes can cope with the increased oxidative burden induced by the high levels of cytochrome P450 activity. The second implication is that the elevated GST activity in the FUMOZ R is a biologically relevant adaptation, as it plays a significant role in pyrethroid resistance. It shows that even if cytochrome P450s are active, if a mechanism is not in place to cope with the oxidative burden, that monoxygenase detoxification alone may not be able to be cope with pyrethroid intoxication. The system may therefore be overwhelmed not only be insecticide-induced toxicity, but by the cytochrome P450-induced oxidative burden. This may explain the role of increased GST activity in the FUMOZ R strain. Although no GSTs were associated with pyrethroid resistance in a QTL analysis (Wondji *et al*, 2005), evidence presented earlier in chapter 2 demonstrated increased GST activity in this strain compared to the susceptible FANG strain. This is congruent with earlier findings showing increased GST transcription (Christian *et al*., 2011) and increased GST activity in the resistant *An. funestus* strain. Although the GSTs may not play a direct role in pyrethroid metabolism, it is conceivable that they may play a role in protection against pyrethroid-induced oxidative stress. The increased GST activity may have evolved originally to cope with the increased ROS production associated with the elevated cytochrome P450 activity.

It would be wise to consider why catalase synergism is so much more efficient than SOD synergism. There are several potential answers to this question. The first has to do with the diversity of the enzyme families. An examination of the UniProt database (www.uniprot.org) indicates that four catalases have been annotated in *An. gambiae*. Of these four catalases, two are based on homology and the remaining two have been proved to exist as transcripts. Seven SODs have been annotated in *An. gambiae*; four have been identified by homology and three have been proved to exist as transcripts. Details of these

proteins are provided in Appendix 5. It therefore appears that there are more individual SOD proteins than there are catalases, all of which may have differential sensitivities to the inhibitors. Furthermore, DDC specifically inhibits Cu/ZnSODs, and MnSODs are unaffected by this synergist. Therefore DDC synergism does not completely negate SOD activity as MnSODs can still remain active at inhibiting concentrations of Cu/Zn SODs (Iqbal and Whitney, 1991) and the remaining SOD activity provided by this class may explain why DDC is the worst-performing inhibitor.

The second issue has to do with the cellular and tissue location of the respective enzymes. SODs are found in the mitochondria, cytosol, as well as extracellularly and this would compound the difficulty in synergising these enzymes. Catalases are found solely in peroxisomes, and therefore enzyme inhibition would be simpler. Most importantly, the synergist was administered orally and catalases are abundant in the midgut. This makes the synergist delivery system well suited to inhibit the catalases present in the midgut.

This study has demonstrated that oxidative stress plays a vital role in the life history and expression of insecticide resistance of the *Anopheles* species tested. All insecticides tested in this study induced a significant oxidative burden. The two insecticides that induce the greatest oxidative stress (permethrin and malathion) also had the most deleterious effect on subsequent longevity.The capacity to cope with oxidative stress is amplified in insecticide resistant *An. arabiensis* and *An. funestus*, with the primary insecticide resistance mechanism playing a crucial role in the degree of amplification. Elevated cytochrome P450 activity appears to be the strongest driver of increased oxidative stress defence, as demonstrated in *An. funestus*. This may also be due to biological differences between species, but the data presented in this study appears to fit the hypothesis. Biochemical studies on F1 progeny of wild-caught *An. funestus* females suggest that this effect is not a laboratory artefact. Synergism of oxidative stress enzymes can affect the insecticide resistance phenotype, particularly in situations where only metabolic resistance is present. Taking into account the synergist delivery system, catalase appears to play the biggest role in the resistance phenotype. This study also provides more detail concerning of the nature of pyrethroid resistance in *An. funestus*.

Chapter 5: The effect of multiple blood-meals on the life history and expression of insecticide resistance in *Anopheles arabiensis*

5.1 Introduction

5.1.1 Blood feeding behaviour in mosquitoes

The haematophagous nature of mosquitoes is why they are one of the most studied dipterans. It is their obligate sanguinivorous nature that makes them of such medical significance. Mosquitoes are therefore obligate parasites and a discussion on their feeding has to begin with why this particular behaviour is necessary.

Haematophagy in dipterans has a variety of functions. In the *Culicidae*, however, blood is primarily required for reproductive functions. As described in Chapter 3, the requirement for blood is largely dependent on larval teneral reserves. The female that emerges from a well nourished larva will require fewer blood-meals to initiate a gonotropic cycle (Attardo, 2005). The primary factor driving blood-feeding, however, depends on whether the mosquito is autogenous or anautogenous.

Anautogeny is the requirement of supplemental protein in order to initiate a gonotropic cycle, specifically oogenesis. Conversely, autogenous species can produce their first batch of eggs without a protein meal (Lane and Crosskey, 1993). The protein meal is not necessarily a blood-meal, because for non-haematophagous flies, such as *Drosophila*, protein is also essential for egg production (Sang and King, 1961). The expression of either autogeny or anautogeny can be obligate or facultative depending on the species (Hansen *et al*., 2004). Anophelines tend to be obligately anautogenous (Clements, 2000) while *Ochlerotatus atropalpus*, an invasive species of *Aedes,* is completely autogenous (Telang and Wells, 2004) There are, however, both *Culex* and *Aedes* species that are facultatively autogenous. The expression of autogeny is dependent on larval environmental conditions, and often the mating status of the adult as well. Typically, autogeny in *Culex pipiens* is driven primarily by the quality of larval teneral reserves. For *Aedes taeniorhynchus*, mating is needed for autogeny when the females have low teneral

reserves. When autogeny is induced by mating, the egg batch is larger than a mating independent batch (O'Meara, 1979).

An obvious question may be which of the two reproductive strategies is the most successful. A mathematical model suggests that autogeny is more advantageous than anautogeny under a specific set of parameters. These include low survival after blood feeding and a high ratio of fecundity of the first autogenous to anautogenous egg batch (Tsuji *et al.*, 1990). Anautogeny, however, usually results in larger egg batches (Reisen and Milby, 1987) and results in increased host contact, making them more efficient disease vectors (Scott and Takken, 2012).

5.1.2 Digestion of blood and the reproductive consequences

Regardless of whether mosquitoes are autogenous or anautogenous, their digestive processes remain the same. Adult female mosquitoes feed on both blood and nectar but the meals are differentially partitioned. Blood typically goes to the midgut, while sugar goes to the ventral portion of the diverticulum, the organ commonly known as the crop (Trembley, 1952). The diverticulum is a strange organ, and sugars are released to midgut for digestion where they are digested more slowly than blood. Sugars are typically used to power flight (Clements, 2000). Sugar is more freely available in the environment than blood, although blood is essential for reproduction. What happens, however, when the midgut is partially or completely full with sugar when the opportunity for a blood-meal arises? What occurs is a “switching mechanism” where the blood can be taken to the crop if necessary. Different species have differing capacities for switching. *Aedes* species are more efficient at switching than *Anopheles* or *Culex* species (Day, 1954). This may underlie the feeding habits of the differing species.

When a blood-meal is obtained, the blood generally goes directly to the midgut. The blood-meal ingestion induces epithelial distension of the midgut, forming a transient organ known as the peritrophic membrane. The peritrophic membrane (PM) is a fibrous network onto which proteins and glycosaminoglycans assemble. It is a semi-permeable membrane that plays a role in the defence against pathogens and mechanical injury from

digested particles. It may also play a role in regulating digestion. This is because the PM is a digestive barrier, as digestive enzymes from the midgut epithelium need to cross the PM to the food bolus. Similarly, the products of digestion need to pass through the PM (Villalon *et al*., 2003). The PM may also serve as protective device to prevent damage from reactive oxygen species (ROS) (Toh *et al*., 2010).

The blood-meal is a highly nutritious food source and the primary source of amino acids in the female. Amino acids in the blood-meal are either protein bound or free in the plasma. Free plasma amino acids are rapidly absorbed and metabolised within in 8 hours post blood-meal. Phenylalanine is the bound amino acid most incorporated into eggs. Isoleucine is the single most important amino acid in the egg production process (Chang and Judson, 1977) It has been demonstrated that host blood does not affect isoleucine incorporation into egg protein (Zhou and Miesfeld, 2009). It was previously thought that human blood usually resulted in lower egg production due to lower concentrations of isoleucine (Valle, 1993).

The ingested blood-meal contains the nutrients required to initiate egg development or oogenesis. In late oogenesis there is the accumulation of the vitellus, the supply of nutrients that the egg needs to get to survive until eclosion. This period of vitellus deposition is known as vitellogenesis, or yolk accumulation, and is a period of rapid oocyte development. The major protein deposited in the vitellus is the protein vitellin, which can constitute up to 70-90% of the egg. Vitellin is derived from vitellogen. Vitellogen (Vg) synthesis is a rapid process which requires a large reorganisation of the fat body, the protein source to the end product, the ovum. In *Aedes aegypti*, Vg synthesis is most intense 27 hours post blood-meal. Vg is broken down by lysozyme, whose activity is highest 30 hours post blood-meal (Valle, 1993).

When female mosquitoes take a *Plasmodium* infected blood-meal, they not only take up nutrients, but the parasite as well. There is an interaction between reproductive processes and the parasite. It has been shown that parasite infection induces a reduction in Vg mRNA abundance (Ahmed *et al*., 2001). This may be a part of parasite manipulation of the host. It has been noted that *Plasmodium* infection reduces mosquito fecundity (Hogg

and Hurd, 1995, Hopwood *et al.*, 2001). Lipophorin and Vg, both nutrient transport proteins, reduce the parasite killing efficiency of the antiparasitic factor Telomerase protein component 1(TEP 1) (Rono *et al.*, 2010). This results in increased parasite efficacy in the mosquito host.

5.1.3 The biological response to a blood-meal

The ingestion of parasites while taking blood-meals is an obvious consideration for most mosquito biologists. This is, however, not the only danger associated with the act of blood-feeding. One of the key dangers in the search for a blood-meal is the risk of being killed by either predators, or during the act of blood feeding (Roitberg *et al*., 2003, Scott and Takken, 2012). When the female does take a blood-meal, it also presents a metabolic challenge to the female.

Haemoglobin is the primary source of protein in haematophagous insects (Pascoa *et al.*, 2002). Dietary haemoglobin is a source of iron and haeme, which are released by haemoglobinolysis. Haeme is a nutrient, but is toxic in its free form. This is due to its' ability to generate reactive oxygen species (ROS) and induce lipid peroxidation. Iron forms ROS primarily through the Fenton reaction (Toh *et al*., 2010). The catabolism of erythrocytes results in high concentrations of haeme not often encountered in vertebrates. Therefore mosquitoes require a variety of defences against the toxic effects of haemoglobin catabolism that vertebrates do not have (Toh *et al*., 2010).

The first line of defence against haeme is a containment system. The PM provides a physical barrier for haeme. Haeme is inserted into crystalline structures and bound by haeme-binding proteins. Haeme can also be catabolised by haeme oxygenase and GSTs (Toh *et al*., 2010).

Haemoglobin is not the only human host derived factor of interest. Various other host derived blood factors are able to trigger signal transduction. Transforming growth factor beta (TGF-β) is one such factor. TGF-β1 in mammals promotes responses that limit parasite growth. The latent form of TGF-β1 is ingested in the blood-meal, and this is

rapidly activated by common products of blood digestion such as haeme and nitric oxide. Latent circulating concentrations of TGF-β1 in high enough concentrations can be biologically active in arthropods. There is extensive cross-talk between mammalian and invertebrate TGF-β proteins, which can have an effect on invertebrate signal transduction systems (Pakpour *et al*., 2013). Another important host protein to consider is insulin.

5.1.4 Insulin and the mosquito

There are two major reasons why insulin is of interest in mosquito biology. Malaria infected patients are very often hyperinsulinanaemic, and therefore mosquitoes feeding on these patients would have a source of excess dietary insulin (White *et al*., 1987). More importantly though, is the conservation of the insulin signalling pathway. Although human insulin would not be the primary initiator of this pathway, like TGF-β1, the possibility of it being able to modulate the insect signal transduction cascade is a real one (Luckhart and Riehle, 2007).

Invertebrates themselves do not produce insulin. Various invertebrate homologues do, however, exist. The existence of seven insulin-like proteins (ILPs) has been confirmed. In *Drosophila* an insulin receptor, known as the DIR, that can bind human insulin but no ILPs have been discovered. A similar receptor, the mosquito insulin receptor (MIR), has been confirmed in *Aedes* (Luckhart and Riehle, 2007). Why would such receptors exist and what function would insulin and ILPs play in invertebrates?

Firstly, it must be noted that insulin does not perform the same role in invertebrates as it does in vertebrates. In *Drosophila*, insulin does not play a role in glucose uptake, affect glycogen concentration or lipid synthesis. It does, however, increase glucose oxidation and lactate oxidation, thus providing growth precursors (Wu and Brown, 2006).

The reduction of DIR expression in *Drosophila* resulted in an 85% increase in lifespan (Tatar *et al*., 2001). A possible explanation for this is that human insulin increased the concentration of reactive oxygen species (ROS) (Kang *et al*., 2008) and without the receptor to bind to, this effect was not transduced. It is interesting to note that this effect

was still apparent 24 hours post blood feeding in *Aedes aegypti*, but disappeared shortly afterwards. Receptor transcripts and proteins were not evident in other life stages (Riehle and Brown, 2002).

A body of evidence exists that exogenous insulin can manipulate invertebrate signalling pathways. Excess insulin has been shown to decrease the lifespan of *An. stephensi* (Kang *et al.*, 2008) due to the induction of oxidative stress. Conversely, studies in the firebug *Pyrrhocoris apterus* showed that porcine glucagon is capable of reducing paraquat-induced oxidative stress. This is mediated by reducing lipid peroxidation and increasing the concentration of reduced glutathione (Alquicer *et al.*, 2009). Bovine insulin has been shown to be able to stimulate ovarian ecdysteroid production in mosquitoes (Riehle and Brown, 1999).

Exogenous insulin interacts with ILPs. At high concentrations, insulin results in the formation of reactive nitrogen species (RNS), oxidative stressants which result in parasite control. At low concentrations, it spreads and enhances the effects of ILPs on immunity and reproduction (Luckhart and Riehle, 2007). It is important to note that high concentrations of insulin are immunosuppressive in both mammals and invertebrates. Insulin-fed *Anopheles* induce higher levels of *Plasmodium* infection (Beier *et al*., 1994).

ILPs have a variety of biological functions. Overexpression of ILPs in *Drosophila* results in bigger flies (Wu and Brown, 2006). They also play a role in the control of metabolic activity and reproductive physiology. This is accomplished by regulating sugar concentration and ovarian development (Luckhart and Riehle, 2007).

5.1.5 Blood and Longevity

The study by Kang *et al.* (2008) was the first to examine the effect of insulin on mosquito longevity. The study concluded that the insulin-induced decrease in lifespan is due to oxidative stress (Kang *et al*., 2008). As mentioned before, small changes in mosquito lifespan have large consequences for competence (Garrett-Jones and Shidrawi, 1969). It is therefore possible that other blood-borne components or blood as a whole,

have an effect on longevity. It has been demonstrated previously that blood and sugar combined result in a better longevity than sugar alone or blood only (Gary and Foster, 2001). The issue of blood and longevity in anophelines, however, has not been carefully dissected and further research is needed into the effect of blood on longevity.

5.1.6 Blood and insecticide resistance

One of the most crucial considerations in any vector control programme is insecticide resistance. It poses a major threat to control efforts, and any factor that is capable of modulating insecticide resistance is worth examining. As such, blood-feeding is an important consideration as a body of research exists about the effect of blood on resistance.

The observation that blood has an effect on the expression of insecticide resistance is not a new one, and has been observed in both the laboratory and the field. It has been noted as early as 1946 that resistance levels fluctuate with blood-feeding, with resistance levels being highest 24 hours after the blood-meal (David and Bracey, 1946). This observation was also noted in a later study. While examining the effect of blood-feeding on *Aedes aegypti* resistance patterns it was noted that DDT and dieldrin resistance was higher 24 hours post blood-meal compared to 3 hours. A similar pattern was observed in *An. stephensi*. This species also demonstrated a cyclic peak in insecticide resistance. Again, resistance was highest 24 hours post blood-meal, but dropped 48 hours post blood-meal (Barlow and Hadaway, 1956). This effect is not species-specific as it has been observed in *An. funestus* (Spillings *et al*., 2008) and in *C. pipiens*, *Culiseta melanura* and *Ae. aegypti* (Eliason *et al*., 1990). In these species it was demonstrated that their susceptibility to aerosols varies with blood-meal digestion. The greatest variability in susceptibility occurred when undigested blood was present (Eliason *et al*., 1990). In this study, as confirmed by Spillings *et al.* (2008), it was demonstrated that the volume of blood does not affect the change in susceptibility, suggesting that this effect is not mediated by vigour tolerance. The three different species differed as to when they displayed their lowest insecticide susceptibility post blood-meal. *Aedes aegypti* resistance

peaks 24 post feed, *C. pipiens* 48 hours post feed and *Cs. melanura* 72 hours post feed. It is interesting to note that blood-feeding did not have an effect on the expression of malathion resistance in *C. pipiens* (Eliason *et al*., 1990).

Field evidence also exists for the effect of blood-meals to prove that this effect is not a laboratory artefact. In South Africa, it was demonstrated that *An. arabiensis* that had taken a blood-meal had increased survival after DDT exposure (Sharp *et al*., 1990). It was also observed that the effect of ultra low volume resmethrin oscillated with gonotrophic cycle. It was concluded that this oscillation arose from blood-meal induced changes to susceptibility to insecticides (Reiter *et al*., 1990).

The previous studies concluded that the changes in insecticide resistance were not due to vigour tolerance (Reiter *et al*., 1990; Spillings *et al*., 2008). This raises the question of how a blood-meal changes the susceptibility of a mosquito to an insecticide. This was the focus of a study by Halliday and Feyereisen (1987). Their study confirmed that vigour tolerance is not the underlying mechanism as the female lost a third of her blood-meal weight 3 hours post blood-meal. They did not find any changes in DDT metabolism or distribution. Despite the abdominal distension, they did not find any changes in cuticular penetration either. What they did find was that vitellogenesis modifies DDT distribution. The accumulation of DDT was highest at maximal vitellogenesis, 72 hours post blood-meal. When oocyte development was almost complete, the amount of DDT in ovaries and the fat body decreased (Halliday and Feyereisen, 1987). Despite their elegantly designed experiment, no conclusion was made about how blood influenced DDT toxicity.

5.1.7 Senescence and insecticide resistance

Senescence is the increased likelihood of death as an organism ages. Until 2007 it was believed that mosquitoes display constant mortality, and have an equal likelihood of death throughout their lives (Styer *et al*., 2007). Two important events are associated with mosquito senescence. The intrinsic incubation period of the *Plasmodium* parasite is between 11 to 14 days, therefore a young mosquito will never be infective (Day, 1954). Secondly, it is an established fact that as a mosquito senesces, it becomes more

susceptible to insecticides. Again, this observation is not new. As early as 1946 a "progressive decline in resistance with age" was observed (David and Bracey, 1946). Since then, a variety of studies have been made about the resistance phenotype in the ageing mosquito.

Malathion resistance was shown to decline in *An. stephensi* (Rowland and Hemingway, 1987). DDT resistance was shown to decline in *An. gambiae* (Lines and Nassor, 1991), and a similar decline was shown in deltamethrin resistance (Jones *et al*., 2012a), and another study on *An. gambiae* in Côte D'Ivoire also showed increasing susceptibility to insecticides with age (Chouaibou *et al.*, 2012). It has even been suggested that that the effect of age is more important in the expression of insecticide resistance than larval nutrition (Kulma *et al*., 2013).

It is important to note that Lines and Nassor (1991) state that "results suggest that in the case of *An. gambiae* in Zanzibar, the decline of DDT resistance with age is not an effect of undernourishment, but also occurs in natural populations taking regular blood-meals". We have a body of research stating that insecticide susceptibility increases with age, but another body of research states that a blood-meal reduces insecticide toxicity, thus increasing the resistance of a female mosquito. A female is likely to take multiple blood-meals during her lifetime (Scott and Takken, 2012) therefore, what effect does that have on the increasing levels of susceptibility in the senescing mosquito? Although the previously mentioned studies did not take blood-feeding into account, with none of the experimental mosquitoes being offered a blood-meal, the effect of blood-meals on the resistance phenotype of the ageing mosquito has to be considered.

The few studies that have considered the effect of blood-meals are in agreement with Lines and Nassor (1991) that blood-meals do not make a difference. A study of *An. gambiae* and *An. stephensi* did not observe a significant difference in the permethrin-induced mortality of 10-day old females that had 3 blood-meals compared to those that had none (Hodjati and Curtis, 1999b). A similar study performed using *An. gambiae* and *Ae. aegypti* came to the same conclusion, although it is important to note that in this study only a single blood-meal was offered to 14-day old mosquitoes (Rajatileka *et al*., 2011).

Glunt *et al.* (2011) also agree with the findings, but also acknowledge that blood-meals increase permethrin resistance of *An. gambiae* (Glunt *et al*., 2011).

5.1.8 Enzymology and resistance

It was first proposed by Rowland and Hemingway (1987) that the observed decrease in resistance with age was correlated with a concomitant decrease in detoxification enzyme activity. The study proposed that decreasing malathion resistance rather be considered an increased susceptibility to the insecticide due to an age-induced reduction of malathion carboxylesterase activity. The decreased activity was attributed to lower amounts of enzyme rather than a change in its' kinetic properties (Rowland and Hemingway, 1987). It is important to note that this study also not only reported that increased malathion susceptibility in RR, RS and SS mosquitoes with age, but that the amount of soluble protein decreased in resistant mosquitoes but remained constant in susceptible mosquitoes (Rowland and Hemingway, 1987).

Several studies looking at enzyme kinetics with age corroborate this work. Studies of *Ae. aegypti* observed GST activity with age and without blood-meals. It was demonstrated that GST activity reached a plateau at 5 days, remained constant for 33 days and declined by 31% in the senescent adult. Age related changes in GSTs were located in the abdomen, where over 50% of the activity occurred, but it was uncertain whether the GST activity that declined was a general phenomenon or only due to decreased activity of a subset of the enzyme population. The activity drop in males and females were similar. As with Rowland and Hemingway (1987), this study observed that the change was in the specific activity (V_{max}) of the enzyme rather than the affinity (K_m) (Hazelton and Lang, 1983).

A similar study showed that while esterase activities in susceptible strains were lower than in resistant strains, they were stable throughout their life, while activity declined in resistant strains. It was also demonstrated that GST activity declines continuously, with the most marked effect being in resistant individuals (Mourya *et al*., 1993). A study in *An. arabiensis* and *An. gambiae* demonstrated that cytochrome P450 activity declined

with age. Furthermore, an examination of the susceptible *An. gambiae* strain Kisumu showed no significant difference in P450 activity levels of blood-fed compared to non blood-fed females (Chen *et al*., 2008).

It would therefore be simple to conclude that mosquito insecticide resistance declines with age, regardless of blood-feeding status. This has been suggested as early as 1987 with statements like "there was no indication that resistance would cease to decline in mosquitoes that approached infective age" (Rowland and Hemingway, 1987). This led to suggestions that ageing can partially restore vector control efforts (Chouaibou *et al*., 2012, Jones *et al*., 2012a). It has also led to speculation that age-targeted strategies can be used, as a low dosage of insecticide could target older, more susceptible females without putting a selection pressure on younger mosquitoes (Glunt *et al*., 2011).

There are, however, several issues that are not neatly resolved. Spillings *et al.* (2008) demonstrated that a single blood-meal increased the permethrin resistance of resistant *An. funestus*, but does not make susceptible females any less sensitive to the insecticide. This suggests that the blood-feeding effect does not occur uniformly in all mosquitoes. How does *kdr* affect this response to blood-feeding? What happens when females are allowed more than 3 blood-meals? This study aimed to carefully dissect the effect of age and blood-feeding on the expression of insecticide resistance as well as to examine the effect of blood and blood components on the longevity of laboratory strains of *An. arabiensis*. These questions have far reaching implications for the efficacy of vector control efforts.

5.2 Materials and Methods

5.2.1 Mosquito strains

This experiment was performed on the *Anopheles arabiensis* strains SENN and SENN DDT. Descriptions of the strains and the conditions they were raised under can be found in chapter 1.

5.2.2 Bioassays of mosquitoes with age

5.2.2.1 Blood feeding bioassays

Adult females drawn for bioassays were differentiated by 3 different nutritional regimes. The first cohort was only allowed 10% sucrose for the duration of their lives. This cohort, called the 0 blood or sugar cohort, constituted the control. The second cohort was allowed a single blood-meal at either the age of 3,7,11, 15, 18 or 21 days. This cohort, called the 1 blood cohort, constituted the single blood-meal cohort. The final cohort was fed a blood meal at the age of 3, 7, 11, 15, 18 and 21 days. At each age point, a sub-sample of each population was removed for bioassays. Therefore, each sample removed at 7 days of age had taken 2 blood-meals; each sample removed at 11 days had taken 3 blood-meals and so forth. This group, called the multiblood group constituted the cohort that had multiple meals. Throughout their lives, males and females were kept together and allowed to mate. Females were allowed to oviposit twice weekly for the duration of the experiment.

At the ages of 3, 7, 11, 15, 18 and 21 days from emergence, samples were drawn from each dietary cohort. Those that were allowed blood in their diet were fed to repletion on a human volunteer. Four hours after feeding, they were exposed to either DDT, permethrin, deltamethrin or malathion according to standard WHO procedures (WHO, 2013). After exposure, the females were allowed access to 10% sucrose solution and mortality was scored 24 hours post exposure. All exposures took place at $25^{o}C \pm 2^{o}C$ and between 75-85% humidity. An unexposed tube constituted an environmental control. No correction for mortality was performed as control mortality never exceeded 10%. A summary of the sample sizes (n) in this study is given in table 5.2.1.

Table 5.2.1: Sample sizes of *Anopheles arabiensis* (SENN DDT) insecticide treatments by age.

Treatment	Age	DDT	Permethrin	Deltamethrin	Malathion
Sugar Fed	3 days	714	696	695	813
	7 days	476	464	463	543
	11 days	476	464	463	542
	15 days	476	464	463	-
	18 days	476	464	463	-
	21 days	476	464	463	-
Single Blood Meal	3 days	714	696	695	813
	7 days	476	464	463	543
	11 days	476	464	463	542
	15 days	476	464	463	-
	18 days	476	464	463	-
	21 days	476	464	463	-
Multiple Blood Meals	7 days	476	465	463	543
	11 days	476	464	463	543
	15 days	476	464	463	-
	18 days	476	464	463	-
	21 days	476	465	463	-
Total:		**8568**	**8354**	**8335**	**4882**

5.2.2.2 Insulin bioassays

Insulin bioassays were performed in a similar manner to the blood-feeding bioassays described in 5.2.2.1. For this experiment, the 3 dietary treatments were as follows: the first treatment was fed a solution of 10% sucrose supplemented with 25mM.l^{-1}.HEPES buffer pH 8.2 throughout their lives and constituted the control cohort. The second treatment was supplied with 10% sucrose solution supplemented with 3.4X10^{-4}µM active recombinant human insulin (Sigma I2643) reconstituted in 25mM.l^{-1}.HEPES buffer pH 8.2, a concentration of insulin averaged between the minimum and maximum serum insulin concentration as described by Kang *et al.,* (2008). The third treatment was supplied with the same concentration of insulin as the second group, but the insulin was denatured by boiling at 100^{o}C for 20 minutes prior to addition to the sucrose solution. This was to determine whether any effects observed were due to the hormonal activity of

insulin or due to the presence of the insulin protein. Sucrose-Insulin solutions were changed daily.

Only females were used in these bioassays, as the aim of these experiments was to determine the effect of insulin as a blood component, and only females would be exposed to insulin. Adults used in this experiment were not allowed access to blood. As in blood-meal bioassays, standard WHO DDT bioassays were performed at the ages of 3, 7, 11, 15, 18 and 21 days. Mortality was scored 24 hours post experiment and an unexposed cohort constituted an environmental control. As mortality in this control group never exceeded 10% no correction was made for mortality.

5.2.2.3 Age delayed bioassays

The aim of this experiment was to determine whether the effects on the expression of resistance observed were short term and related to the presence of the blood-meal, or whether the multiple blood-meals had a lasting effect on the phenotype. To do this, an age-delayed bioassay was performed. For this experiment, three nutritional regimes were followed: sugar fed, single blood-meal and multiple meals. The blood-fed cohorts were treated as described in section 5.2.2.1. In this experiment, after a 15 day meal the multiblood cohort was split into 2 groups. The first group, called the age delayed group, received no further blood-meals. The remainder of the group were allowed a final blood-meal at the age of 18 days. Standard WHO bioassays as described in 5.2.2.1 for the insecticides DDT, permethrin and deltamethrin. The mean percentage mortalities of the age delayed group were compared to the standard 18-day exposure group using a 2 sample t-test, with a 95% confidence interval.

5.2.3 Detoxification enzyme analysis

5.2.3.1 Specimen preparation

The objective of the following sets of experiments was to determine the effect of blood-meals on the expression of detoxification enzyme activity. Blood-fed mosquitoes are

usually avoided in biochemical assays as blood-borne components interfere with the assays. To avoid this, when the different nutritional regimes were assayed for enzyme activity, specimens were killed 72 hours after their last treatment. This had the two-fold effect of allowing complete digestion of the blood-meal, and examining a more lasting effect of the dietary treatment in the individual. Therefore, in this experiment, although the same time points are used for the bioassay, chronologically the mosquitoes were 3 days older. For example, in the single point detoxification analysis the mosquitoes examined are called 15 day old, but when assayed they were 18 days old. This is because the naming system reflects their meal rather than their age.

For detoxification assays, all specimens were homogenised in PCR grade water after being killed at -70^{o}C. For all detoxification enzyme analysis 96 individuals were used per age and blood feeding regimen for each strain.

5.2.3.2 Single point detoxification enzyme analysis

For this experiment, female SENN and SENN DDT adults were used. For each strain, individuals were either sugar fed, had a single blood-meal at the age of 15 days or multiple blood-meals until the age of 15 days. Seventy two hours later the individuals were killed and prepared as described in 5.2.3.1. Individuals used in this experiment were not exposed to insecticide prior to exposure.

In this experiment only cytochrome P450 and GST activity was assayed as these are the enzymes associated with DDT and pyrethroid resistance respectively, the phenotypes most significantly affected by blood-meals.

5.2.3.3 Detoxification enzyme activity with age

Spillings *et al.* (2008) demonstrated that the effect of blood-feeding on the pyrethroid resistance phenotype was limited to resistant mosquitoes, and did not make insecticide susceptible mosquitoes any less sensitive to insecticides. Based on similar findings, it was decided to only examine the effect of blood-feeding in the ageing mosquito on the phenotypically insecticide resistant SENN DDT. SENN- DDT males of the same age

were used as a comparison. At the ages of 3, 7, 11, 15, 18 and 21 days SENN DDT females of the three different blood feeding regimes as well as males of the same age were prepared for enzyme assays as described in 5.2.3.1. Cytochrome P450, GST and general esterase activity were determined as described in section 2.2.2.2.

5.2.4 Oxidative stress analysis

To determine whether other factors besides detoxification enzymes played a role in the blood-meal effect, an analysis of oxidative damage as well as the oxidative stress defence capacity of both SENN and SENN DDT females was performed. The aim of the following sets of experiments was to determine whether blood-feeding had differing effects on the oxidative stress defence systems of the two different strains.

5.2.4.1 Determination of oxidative stress by quantification of protein carbonyl content

Protein carbonyl content is a common marker for oxidative stress, and quantification of total protein carbonyl groups is a good measure of oxidative damage. The DNPH derivitivization reaction described by Reznick and Packer (1994), as utilised by Vontas *et al.* (2001), is described in chapter 1. Protein carbonyl groups were quantified by calorimetric spectrophotemetry. A 96-well spectrophotometer (Multiskan Ascent, Thermo Scientific) was used to determine the end point reading at 366 nm and protein carbonyl content was determined as the difference in absorbance of DNPH and HCl treated samples at 366nm with an extinction co-efficient of $220mm^{-1}cm^{-1}$.

In the first experiment SENN and SENN DDT females were used. Females from the three feeding regimes were prepared for harvesting as described in 5.2.3.1. A total of 0.15g (approximately 150 individuals) of starting material per strain and treatment were homogenised in 1ml 50mM sodium phosphate buffer pH 7.5 containing 0.5µg/ml leupeptin, aprotinin and pepstatin and 0.1% Triton X-100. Carbonyl content was calculated as described above. The mean carbonyl content of 3 replicates of each strain treatment was compared using 1-way ANOVA.

The second experiment determined whether insecticide exposure alone and in conjunction with multiple meals had an effect on the carbonyl content of SENN DDT. A sugar fed control cohort was exposed to a standard WHO bioassay at the age of 15 days in the same manner as described in 5.2.2.1. Seventy two hours post blood-meal, each treatment was prepared for SENN/SENN DDT comparison and protein carbonyl content was determined. The mean protein carbonyl content of 3 replicates was compared using 1-way ANOVA.

5.2.4.2 Quantification of defence against oxidative stress

Catalase and glutathione peroxidase activity were determined to obtain a measure of defence against oxidative stress. Details of both techniques are detailed in chapter 1. Catalase assays were based on a method described by Sinha (1972) and glutathione peroxidase activity was based on the method of Vontas *et al.* (2001).

For the catalase assay, cohorts of 3-day old non blood-fed SENN and SENN DDT females were killed 72 hours after preparation. As with the previous experiments; the individuals were 3 days older. For all the other treatments, females were aged and prepared as previously described. A total of 250 mosquitoes were assayed in this experiment. Single mosquitoes were homogenised in 0.1M potassium phosphate buffer pH 7.0. The reaction was performed in 100mM potassium phosphate buffer, with H_2O_2 added to a final concentration of 0.8% or 0.26M potassium dichromate (1:3 solution of dichromate: acetic acid) was added to a final concentration of 0.03 M and was added to this mixture. Absorbance was monitored kinetically at 570nm for 30 minutes. A hydrogen peroxide standard curve was used to determine the amount of hydrogen peroxide consumed. The formula used to calculate the peroxidase activity is detailed in the appendices.

15-day old SENN and SENN DDT females for the glutathione peroxidase activity assays were prepared as previously described. Single mosquitoes were homogenised in 50mM potassium phosphate buffer pH 7.0 and crude homogenate containing approximately 0.1mg of protein was added to a reaction mixture containing 1mM EDTA, 0.2mM

NADPH, 1 unit/ml glutathione reductase and 1mM reduced glutathione in a total volume of 300µl. The reaction was incubated at ambient temperature for 5 minutes. The final reaction was initiated by the addition of 1.5mM t-butyl hydroperoxide. NADPH oxidation was measured kinetically as the decrease in absorbance at 340nm for 15 minutes. Glutathione peroxidase activity was calculated as the gradient of the reaction where the reduction of NADPH was proceeding at linear kinetics. The extinction co-efficient for NADPH at 340nm is $6.22nM^{-1}cm^{-1}$ but an extinction co-efficient adjusted for the pathlength of the 96-well plate was $373nM^{-1}$ $cm-^{1}$. As with the catalase assay, a total of 250 individuals were assayed per treatment.

5.2.5 Longevity

The effect of insulin and multiple blood-meals on the longevity of SENN and SENN DDT females was determined. For all longevity experiments, adults were monitored until all individuals were dead and cadavers were removed daily. Results were analysed using the Kaplan-Meier estimator and the Log-rank test was used as a measure of significance as no data were censored. All confidence intervals were set at 95%.

5.2.5.1 Insulin and longevity

In this experiment, 30 females per strain were used for each dietary treatment. A final concentration of 0.34nM active recombinant human insulin reconstituted in $25mM.litre^{-1}$ HEPES buffer pH 8.0 in 10% sucrose solution constituted the experimental diet while 10% sucrose solution supplemented with 25mM per litre HEPES buffer pH 8.0 constituted the control. Insulin-sugar solutions were changed daily. For each dietary treatment, 3 replicates were performed 3 times, with a total of 9 replicates per treatment.

5.2.2.2 Multiple blood-feeding and longevity

As for the previous experiments, 30 females were used per replicate. Newly emerged SENN and SENN DDT adults were separated by sex to ensure that females were not mated. To one cohort 45 males were placed in a cage to allow mating to take place. This

cohort was known as the mated group. They were allowed blood twice weekly until death. A second group was not allowed access to males during their lifetime, but were allowed blood twice weekly until death. This constituted the unmated group. Both cohorts receiving multiple blood-meals were allowed *ad libitum* access to 10% sucrose throughout the duration of their life. Both cohorts were allowed to oviposit twice weekly.

Two control groups were established. The first was a group of virgin females allowed access to sugar and no access to blood, constituting the sugar fed control. A second group of virgins were also denied access to blood, but were fed with 10% sucrose solution supplemented with 50mM BSA. This constituted the protein/BSA control. This control was to determine whether any changes observed were due to additional dietary protein or due to the specific presence of blood.

5.3 Results

5.3.1 The effects of diet and dietary components on the expression of the insecticide resistance phenotype

5.3.1.1 The effect of multiple blood-meals on the expression of insecticide resistance

Multiple blood-meals had a marked effect on DDT, permethrin and deltamethrin-induced mortality. Throughout the lifetime of the mosquito, those that had multiple blood-meals had significantly lower mortality than their sugar fed counterparts ($p<0.01$ in all cases). Interestingly, for both DDT and permethrin, multiple blood-meals had a particularly sustaining effect on the maintenance of the phenotype with age. For DDT, there was no significant difference in mortality in the multiple-fed cohort between the ages of 3-18 days (1-Way ANOVA: $p=0.36$; $F=1.11$, $df=1$). For permethrin there was no significant difference in mortality in the multiple-fed cohort between the ages of 7-21 days (1-Way ANOVA: $p=0.35$; $F=1.13$, $df=1$). Multiple blood-meals did not have the same effect for malathion resistance. A significant reduction of mortality was noted with a single meal at the age of 3 days (2-sample t-test: $p=0.01$; $t= 3.25$) and with multiple meals at the age of 7 days (2-sample t-test: $p=0.02$; $t=-4.24$). This effect was lost by the age of 11 days, and

no further bioassays were performed as mortality for all three groups at this age exceeded 90%.

Single blood-meals did not have as marked effect on the insecticide resistance phenotype. For all four insecticides, at the age of 3 days, a single blood-meal significantly reduced mortality (2 sample t-test: DDT: $p<0.01$; permethrin: $p=0.02$; deltamethrin: $p=0.02$; malathion: $p=0.01$). During the rest of the female's life, a single blood-meal at a specific age had a variable effect on the expression of resistance. There are, however, notable points at different ages for DDT, permethrin and deltamethrin. At the age of 7 days, a single blood-meal resulted in significantly reduced DDT mortality (2 sample t-test: $p<0.01$; $t=-3.98$). For permethrin, although a single meal at the age of 11 days resulted in significantly reduced mortality (2 sample t-test: $p<0.01$; $t=-5.54$), it resulted in significantly higher than control mortality at the ages of 18 days and 21 days (1-Way ANOVA: $p < 0.05$ $F = 6.35$; $df = 1$). At the same ages, single blood-meals had a significant effect on deltamethrin resistance. At the age of 11 days it resulted in significantly lower mortality ($p=0.03$). Unlike permethrin, however, the single meal significantly reduced mortality at the age of 18 days and 21 days (1-Way ANOVA: $p < 0.05$; $F = 4.98$; $df =1$). These results are reflected in figure 5.3.1.

The effect of multiple blood-meals was also examined by considering how the different diets affect the relationship of mortality with time. When a linear regression analysis was performed on mean mortality at different time points, the strongest linear relationships between time and mortality were always observed where no blood-meals had been taken (DDT: $r^2=0.91$; permethrin: $r^2=0.99$; deltamethrin: $r^2= 0.95$; malathion: $r^2=0.86$). For DDT and permethrin, multiple blood-meals showed the weakest linear relationships of the three treatments (DDT: $r^2=0.67$; permethrin: $r^2=0.79$). Although there is a stronger linear relationship between multiple blood-meals than for single meals ($r^2=0.93$ vs. $r^2=0.89$), the relationship in the multiple blood-meal treatments had a smaller gradient (2.45 vs. 4.37). It is interesting to note that although there was a strong linear relationship in single blood-meal mortality for permethrin ($r^2 =0.93$), the linear relationship with mortality for DDT was equally weak for multiple meals ($r^2=0.67$) and single meals

(r^2=0.65). As only two points existed for multiple meals for malathion, a good linear regression analysis could not be performed. These results are summarised in figure 5.3.2 and table 5.3.1 summarises the statistical differences between these relationships.

Table 5.3.1: 1 way ANOVA comparing insecticide-induced mortality of non-fed *Anopheles arabiensis* SENN DDT females at different ages to their counterparts fed either a single or multiple blood meals.

	DDT	**Permethrin**	**Deltamethrin**	**Malathion**
1 blood	p=0.64 (F=0.23;df=1)	p=0.83 (F=0.05;df=1)	p=0.61 (F=0.26;df=1)	p=0.78 (F=0.09;df=1)
Multiblood	p=0.04 (F=5.16;df=1)	p=0.02 (F=8.56;df=1)	p=0.039 (F=6.76;df=1)	p=0.98 (F=0.00;df=1)

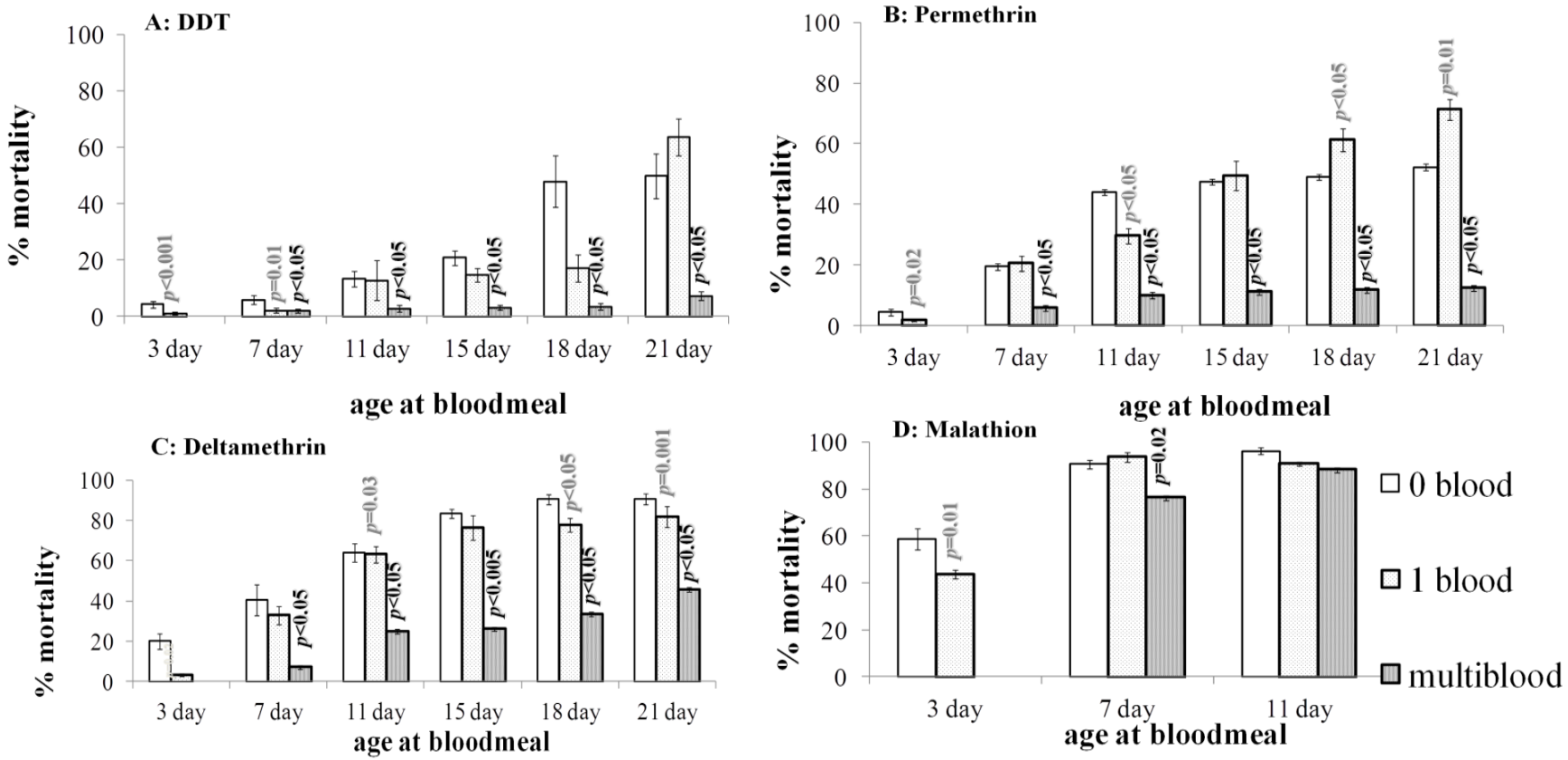


Figure 5.3.1: The effect of blood-feeding on the expression of insecticide resistance in *Anopheles arabiensis* SENN DDT. The insecticide resistance profile with ageing and blood-feeding was examined for DDT ,permethrin, deltamethrin and malathion. P-values are indicated where the treatment differs significantly from the control. For DDT, permethrin and deltamethrin, the multiple blood-meal group always had significantly lower mortality than both the control and single blood-meal cohort. For the DDT treated cohort there was no significant difference in mortality in the multiple blood-meal group between the ages of 3 and 18 days. A single blood-meal at the age of 3 days resulted in significantly lower mortality for all four insecticides. Malathion bioassays were ceased at the age of 11 days as all treatments had reached 90% mortality.

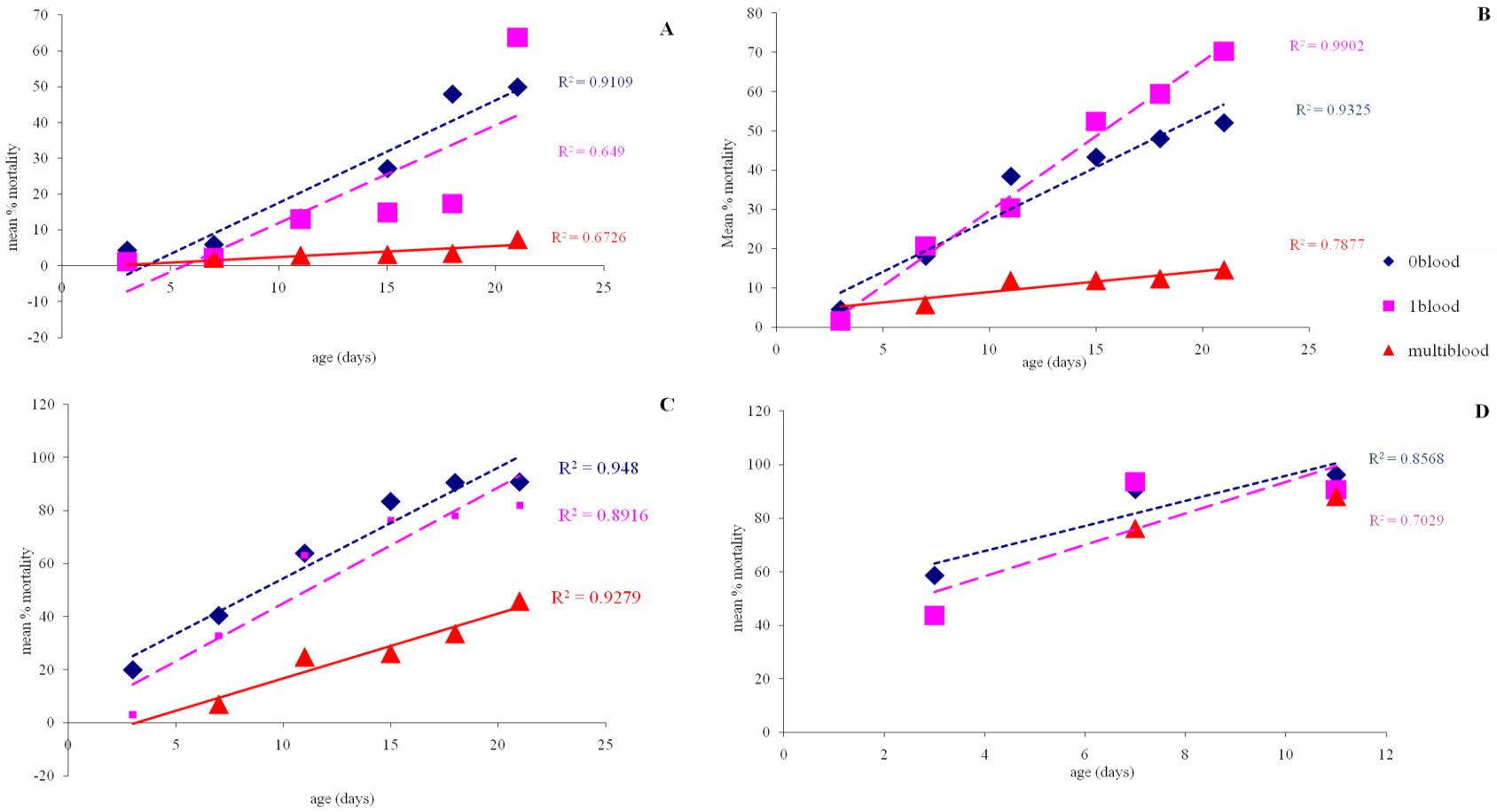


Figure 5.3.2: Linear regression analysis of DDT, permethrin, deltamethrin and malathion induced mortality with age. For DDT (Block A), deltamethrin (Block C) and malathion (Block D), the strongest linear relationship was in the sugar fed controls. For DDT, the linear relationships between age and mortality for the single blood and multiple meal groups were equally weak, but for permethrin (Block B) deltamethrin, and malathion the linear relationships for the single blood-meal groups were strong.

5.3.1.2 Lasting effects of blood-meals on the insecticide resistant phenotype

The previous results suggest that multiple blood-meals may have a lasting effect on the expression of insecticide resistance. This was tested by the delayed exposures described in 5.2.2.3. From the bioassay results described in 5.3.1.1 it is evident that at the age of 18 days, multiple blood-meals resulted in significantly reduced mortality for DDT, permethrin and deltamethrin. When individuals were allowed 4 meals (at the ages of 3,7, 11 and 15 days) prior to exposure at the age of the 18 days, the multiple blood-feeding resistance phenotype was maintained for DDT and permethrin. For these insecticides there was no significant difference in mortality when the females were fed multiple blood-meals up to the age of 18 days, or when they were only allowed meals up to the age of 15 days (DDT:2 sample t-test: $p=0.10$; $t=-1.80$; permethrin: 2 sample t-test: $p=0.88$; $t=-0.15$). This result was not mirrored in the deltamethrin exposures where the delayed exposure group had a significantly higher mortality (2 sample t-test: $p<0.05$; $t=-8.94$). Although the delayed deltamethrin group showed a significantly lower level of mortality than the group that had no blood (2 sample t-test: $p=0.01$; $t=2.85$; $df=15.9$), it did not differ significantly from the single blood-meal group (2 sample t-test: $p=0.91$; $t=-0.12$; $df=21.4$). These results are summarised in figure 5.3.3.

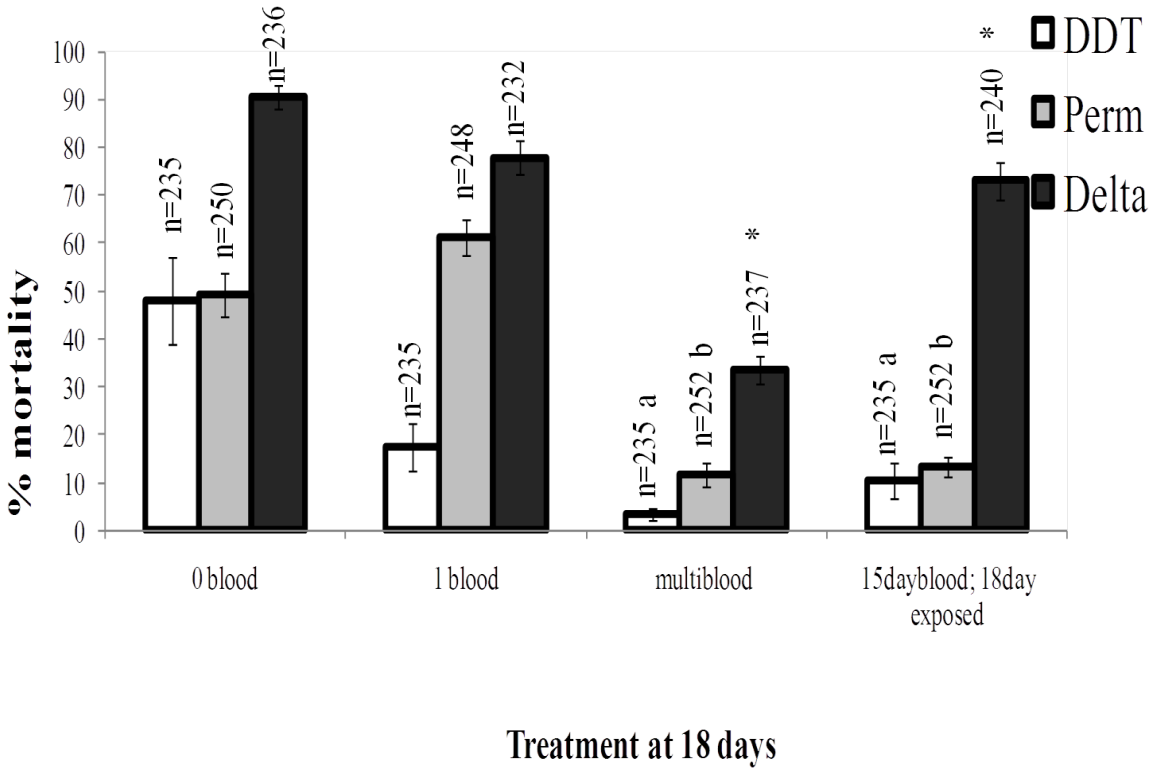


Figure 5.3.3: The lasting effect of multiple blood-meals on the DDT and pyrethroid resistance phenotypes of *Anopheles arabiensis* SENN DDT. When a cohort fed multiple meals until the age of 15 days was exposed to DDT or permethrin at the age of 18 days, there was no significant difference in mortality in the group compared to cohorts of the same age that had multiple meals up to the age of 18 days (a= no significant difference for DDT, b= no significant difference for permethrin). For the same treatment, there was a significant increase in deltamethrin mortality in the group that had their last meal at the age of 15 days (indicated by an asterisk), where this group's mortality did not differ significantly from their counterparts fed only one meal at the age of 18 days. The group fed until 15 days and exposed at 18 days still showed a significantly lower deltamethrin-induced mortality than those that had no blood at all.

5.3.1.3 The effect of insulin on the expression of DDT resistance

The blood component insulin had an effect on the expression of DDT resistance. As depicted in figure 5.3.4, the administration of active recombinant human insulin to the female's diet resulted in significantly reduced mortality at the age of 3 and 7 days (1-way ANOVA: $p=0.02$; $F=4.39$; $df=1$), while denatured insulin did not have the same effect (2 sample t-test: $p=0.09$ $t=-1.68$). Until the age of 11 days, active insulin resulted in significantly lower mortality than denatured insulin (1 way ANOVA: $p<0.05$; $F=13.4$, $df=1$). This effect was lost from the age of 15 days, where no significant differences in mortality existed between active and denatured insulin treatments or between either of the insulin-treated groups and the sugar-fed controls (Generalised linear model: $p=0.64$; $t=-0.46$).

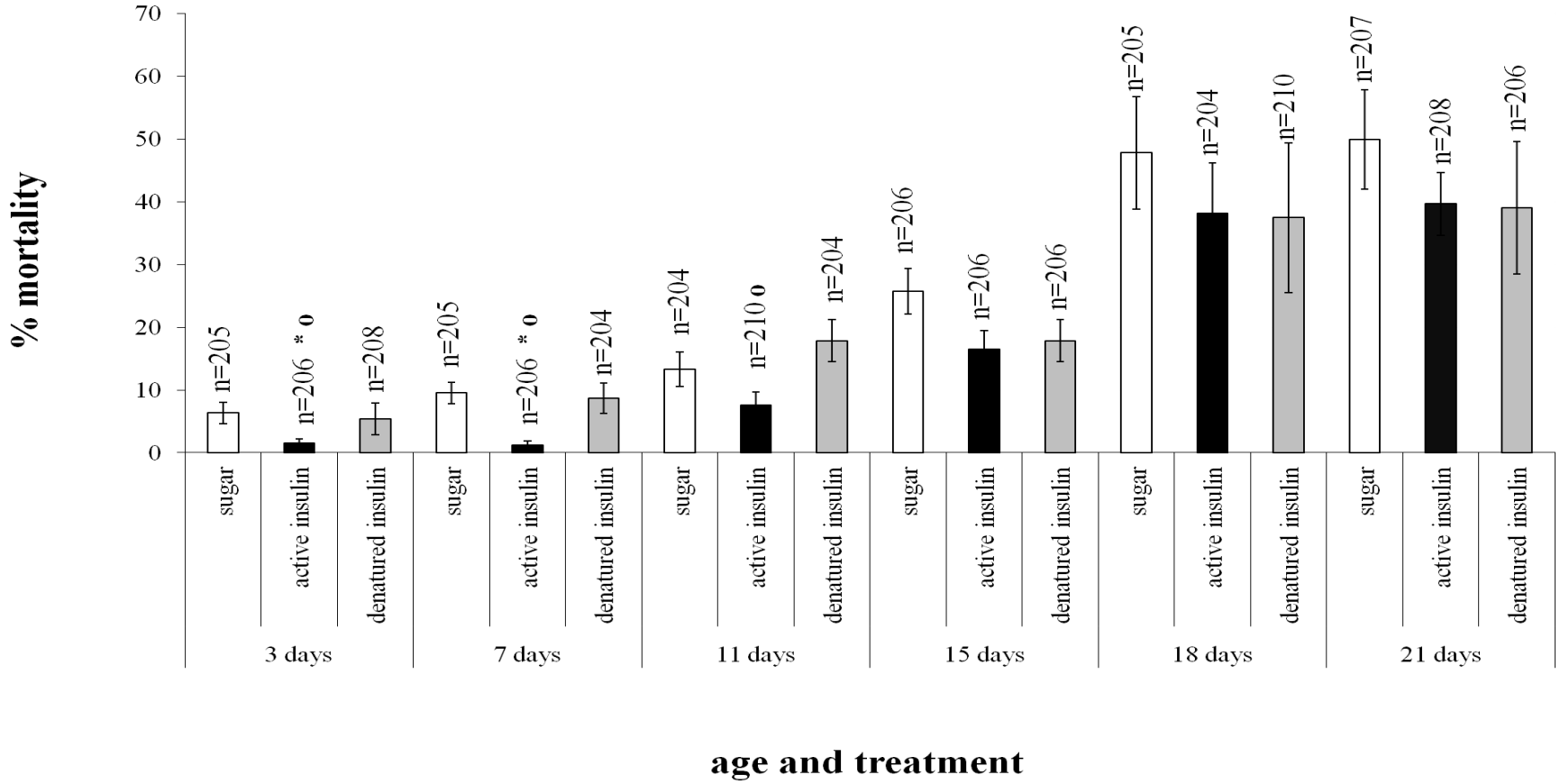


Figure 5.3.4: The effect of exogenous insulin on the expression of DDT resistance in *Anopheles arabiensis* SENN DDT. Active recombinant human insulin or recombinant human insulin that had been heat-denatured was supplied to SENN DDT females in 10% sucrose solution. Active insulin treatment resulted in significantly reduced DDT mortality at the ages of 3 and 7 days (denoted by an asterisk *). Denatured insulin did not have the same effect at any age group. Active insulin resulted in significantly lower mortality than denatured insulin at the ages of 3 to 11 days (difference indicated with a circle (o).

5.3.2 The effect of blood-feeding on metabolic detoxification enzymes

From the previous experiments, it is clear that blood-feeding had a distinct effect on the expression of insecticide resistance. Several experiments were therefore performed to determine how this effect was being modulated. Several questions were asked. Does blood-feeding affect detoxification enzyme activity, and if so what is the effect of multiple meals throughout the lifetime of the mosquito? Secondly, are detoxification enzymes the only mediators of this response? Does the oxidative stress response play a role in the effect of multiple blood-meals on the expression of insecticide resistance?

5.3.2.1 The effect of multiple blood-meals on detoxification enzymes at a single time point

As only the DDT and pyrethroid resistance phenotypes were maintained by multiple blood-meals, it was decided to examine the effect of blood-meals on the activity of GSTs and cytochrome P450s. The age of 15 days was selected as by that age the multiple-meal cohorts had 4 meals, and as observed from the delayed exposure study, there is a clear, lasting phenotypic effect.

When examining the activity of cytochrome P450s, the most pertinent result was that multiple blood-meals had suppressed the enzyme's activity. For SENN DDT, the reduction was significant (2-sample t test: $p=0.01$; $t=2.75$) (Figure 5.3.5A).

An analysis of GST activity demonstrated that SENN DDT females that had multiple blood-meals had significantly higher levels of GST activity than females of the same strain and age that had either no blood (2-sample t-test: $p<0.05$, $t=-7.74$) or a single blood-meal (2-sample t-test: $p<0.05$, $t=-11.88$). Similarly, SENN DDT females that had multiple meals had significantly higher levels of enzyme activity than SENN females that had no blood (2-sample t-test: $p<0.05$, $t=9.22$), a single blood meal (2-sample t-test: $p<0.05$, $t=9.44$) or multiple blood meals (2-sample t-test: $p<0.05$, $t=6.86$). SENN females that had multiple meals had significantly higher GST activity compared to those of the same age that had no blood meals (2 sample t-test: $p<0.05$; $t=-17.31$) or a single meal (2 sample t-test: $p<0.05$; $t=-12.54$). The most interesting result, however, was that SENN

DDT females that had multiple blood-meals had significantly higher GST activity at the age of 18 days than at the age of 3 days (2 sample t-test p=0.01; t=-2.59). In contrast, the GST activity of 3-day old SENN females was significantly higher than 18-day olds that had multiple meals (2 sample t-test: p<0.05; t=-5.99). These results are summarised in figure 5.3.5.

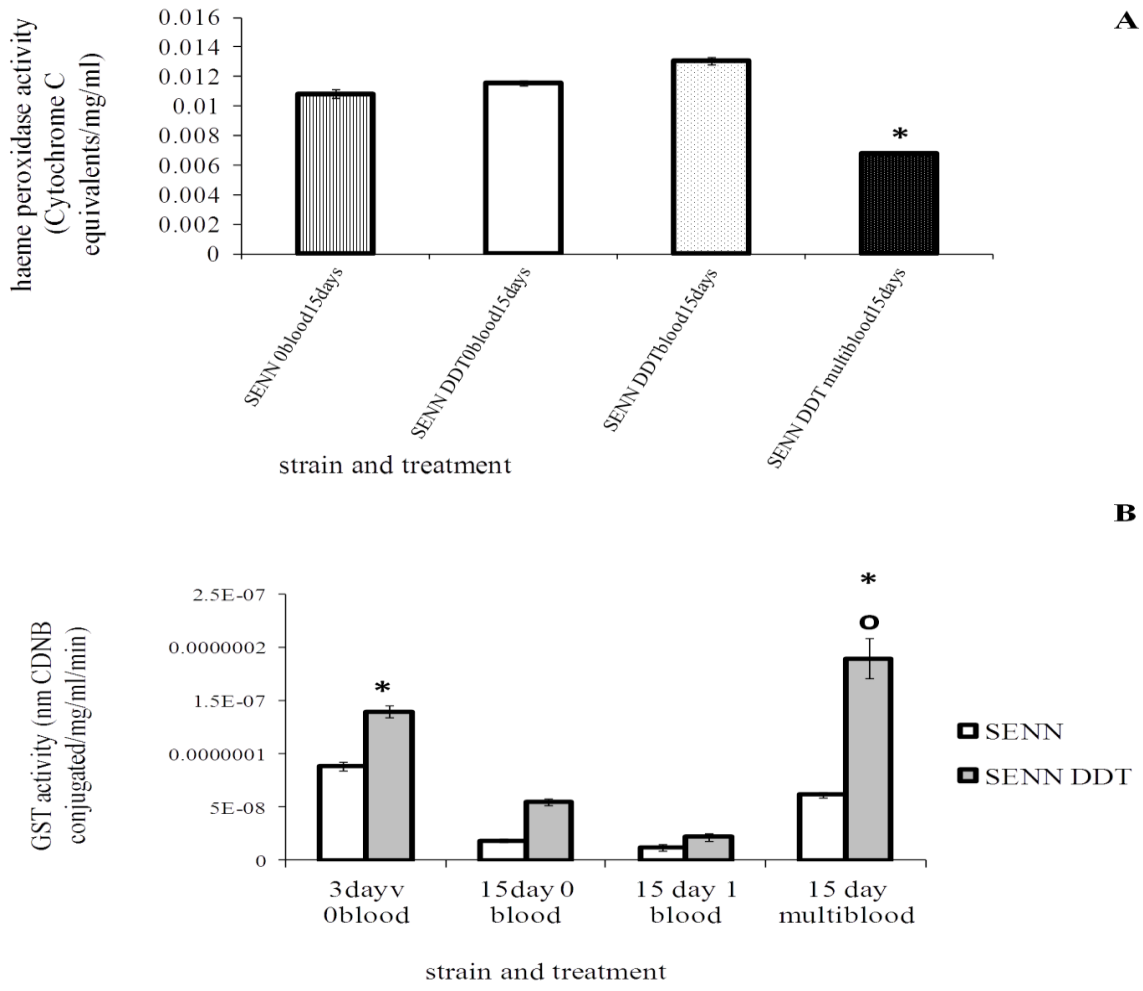


Figure 5.3.5: Metabolic detoxification enzyme activities of 15 day of *Anopheles arabiensis* SENN and SENN DDT adults. Block A represents cytochrome P450 activity and block B GST activity. Multiple blood-meals resulted in a significant reduction of enzyme activity in SENN DDT (denoted by an asterisk). For GST activity, an asterisk denotes a significant difference in enzyme activity between the strains at the same age, while a circle denotes a significant difference in activity of the same stain, but at the ages of 3 and 15 days. Multiple blood-meals induced a significant increase in GST activity in SENN DDT, and the enzyme activity of the multiple meal group was significantly higher than their 3 day old counterparts that had not had blood.

5.3.2.2 The effect of multiple blood-meals on detoxification throughout the lifetime of the adult

From the aforementioned results it is clear that blood-feeding affected detoxification enzyme activity. It is also clear that blood-feeding had a more marked effect on the enzyme activity of insecticide-resistant individuals. For this reason, the enzyme activity of only insecticide resistant individuals was tracked throughout their lifetime. These results are shown in figure 5.3.6.

Block A represents cytochrome P450 activity. Here we see that blood-feeding, either single or multiple meals, suppressed the activity of this enzyme as females that had no blood showed higher levels of enzyme activity throughout their lifetimes (1-way ANOVA $p<0.05$; $F=37.9$; $df=2$). Furthermore, females that had a blood-meal, either single or multiples had significantly lower levels of cytochrome P450 activity than their male counterparts (1-way ANOVA $p<0.05$; $F=13.9$; $df=3$). Only at the ages of 18 (1-way ANOVA $p<0.05$; $F=27$; $df=1$) and 21 days (1-way ANOVA $p<0.05$; $F=32.8$; $df=1$) did the single and multiple blood meal cohorts differ significantly from each other.

Block B represents GST activity. The GST activity of females fed multiple meals declined throughout their lifetime, but even at its' lowest point, it was significantly higher than any point in the life of males or females that had no blood-meals or a single meal (1-way ANOVA $p<0.05$; $F=37.9$; $df=3$).

Block C represents α-esterase activity and Block D represents β-esterase activity. A single blood meal at the age of 3 days significantly increased both α- and β-esterase activity (2 sample t-test: $p<0.05$; $t=5.63$ for α-esterases and $p<0.05$; $t=-4.48$ for β-esterases). Multiple blood meals significantly increased α- and β-esterase activity from the age of 15 days compared to the no blood, single blood and male cohort (1-way ANOVA: $p<0.05$; $F=20.4$; $df=3$ for α-esterases and $p<0.05$; $F=15.8$; $df=3$ for β-esterases).

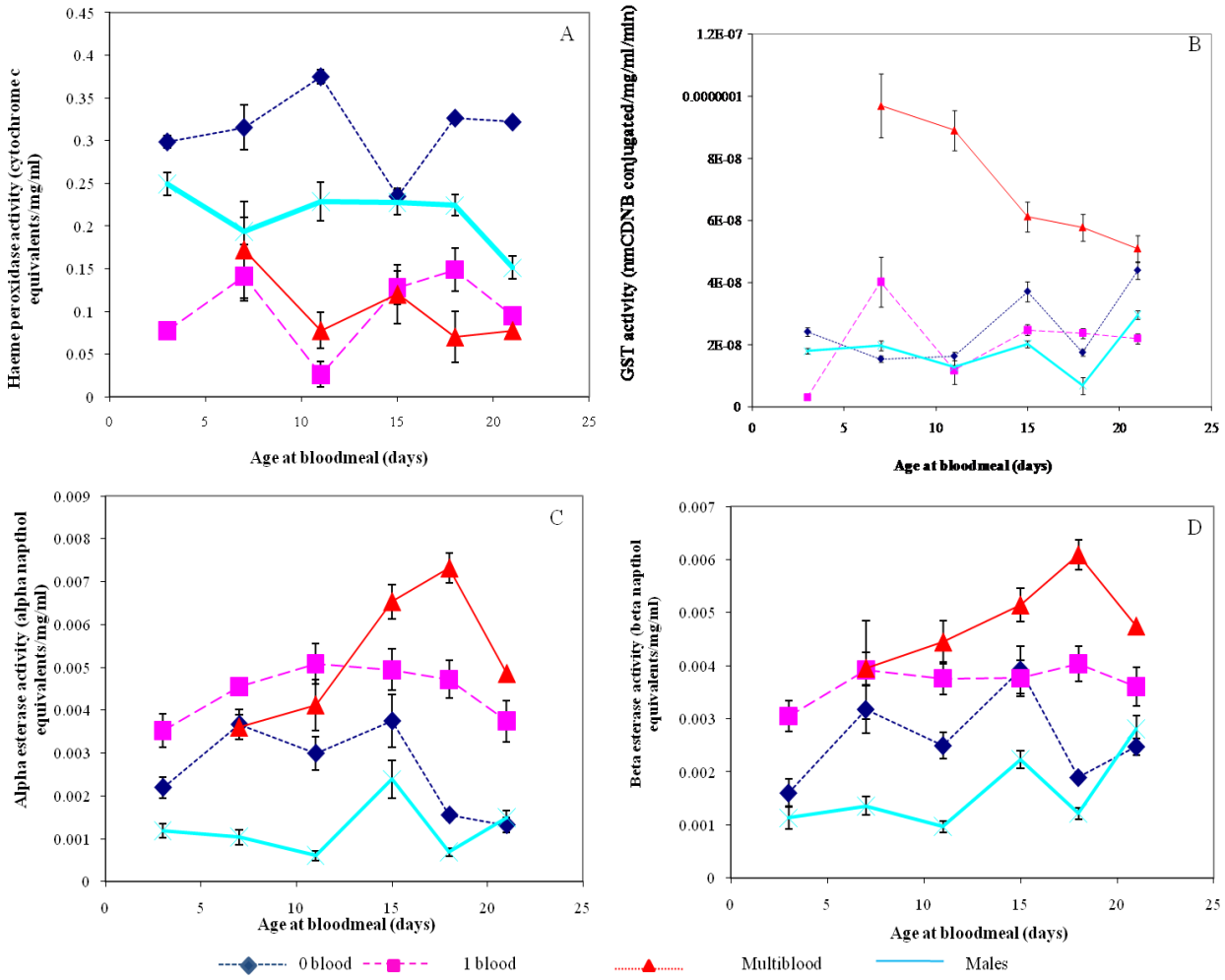


Figure 5.3.6: Metabolic detoxification enzyme activity of *Anopheles arabiensis* SENN with age. The effect of blood-feeding on the activity of cytochrome P450s (Block A), GSTs (Block B), alpha esterases (Block C) and beta esterases (Block D) was tracked. Sugar feeding (dark blue line with diamonds), single blood-meals (pink line with squares), and multiple blood-meals (red line with triangles) were compared to each other with the activity of males (turquoise line with crosses) as a baseline control. For cytochrome P450 activity (Block A) females that had a blood-meal (either single or multiple) had significantly lower enzyme activity either than those that had no blood or males. Only at the ages of 18 and 21 days did the single and multiple blood meal cohorts differ significantly from each other. For GST activity (Block B), although the GST activity of cohorts fed multiple blood-meals decreased throughout their life, it was always significantly higher than any of the other treatments. At the age of 21 days the multiple meal group still had higher GST activity than unfed cohorts 3 days of age. Alpha esterase activity (Block C) tended to mimic the activity of beta esterases (Block D). In both cases, the enzyme activity of the multiple meal cohort increased from the age of 11 days, peaked at the age of 18 days and then declined. For both types of esterases a single blood-meal at the age of 3 days resulted in significant increases in enzyme activity.

5.3.3 Blood-meal modulation of oxidative stress

Analysis of the detoxification enzymes clearly demonstrates that blood-feeding had an effect on enzyme activity. This leads to the next question: is this the sole mediator of the blood-meal effect, or are there other factors involved? The strong effect on GST activity suggests that oxidative stress may play a role as this enzyme superfamily is involved in the oxidative stress response (Vontas *et al*., 2014) (Vontas *et al*., 2001).

5.3.3.1 The effect of blood-meals on the levels of oxidative stress in the adult mosquito

If the oxidative stress response is involved in the mosquito's response to multiple blood-meals, then blood-meals would have an effect on the nett oxidative damage in the insect. Protein carbonyl content is a common and useful measure of oxidative stress in an individual. Figure 5.3.7 represents the study of oxidative stress in the two strains.

In figure 5.3.7 A the nett oxidative damage in SENN and SENN DDT females are summarised. Multiple blood-meals resulted in a significant reduction in oxidative stress in SENN DDT females (1 way ANOVA: p=0.02; F=4.48, df=1) but not SENN females (1 way ANOVA: p=0.09; F=2.72, df=1). SENN DDT females had significantly lower levels of carbonyl than their SENN counterparts that had no blood meals (2 sample t-test; p=0.04, t=2.17), multiple blood meals (2 sample t-test; p=0.01, t=3.31) but not those that only had a single blood meal (2 sample t-test; p=0.12, t=1.64).

As multiple meals had a marked effect on SENN DDT, it was decided to see how blood-feeding interacted with insecticide-induced oxidative stress. The carbonyl content of SENN DDT females that were exposed to DDT alone or in combination with blood meals was examined. DDT exposure alone induced a significant increase in carbonyl content (2 sample t-test p=0.01; t=-4.17), as did a single blood meal (2 sample t-test: p=0.01; t=-2.85). A single blood meal in combination with DDT exposure also induced a significant increase in carbonyl content (2 sample t-test: p= 0.03; t=-2.45). Multiple blood meals alone induced a significantly lower oxidative burden than a single blood meal (2 sample t-test: p=0.04; t=-2.26). Multiple meals with a DDT exposure also induced a significantly

lower level of stress than a single meal in combination with a DDT exposure (2 sample t-test: p=0.03; t=-2.40).

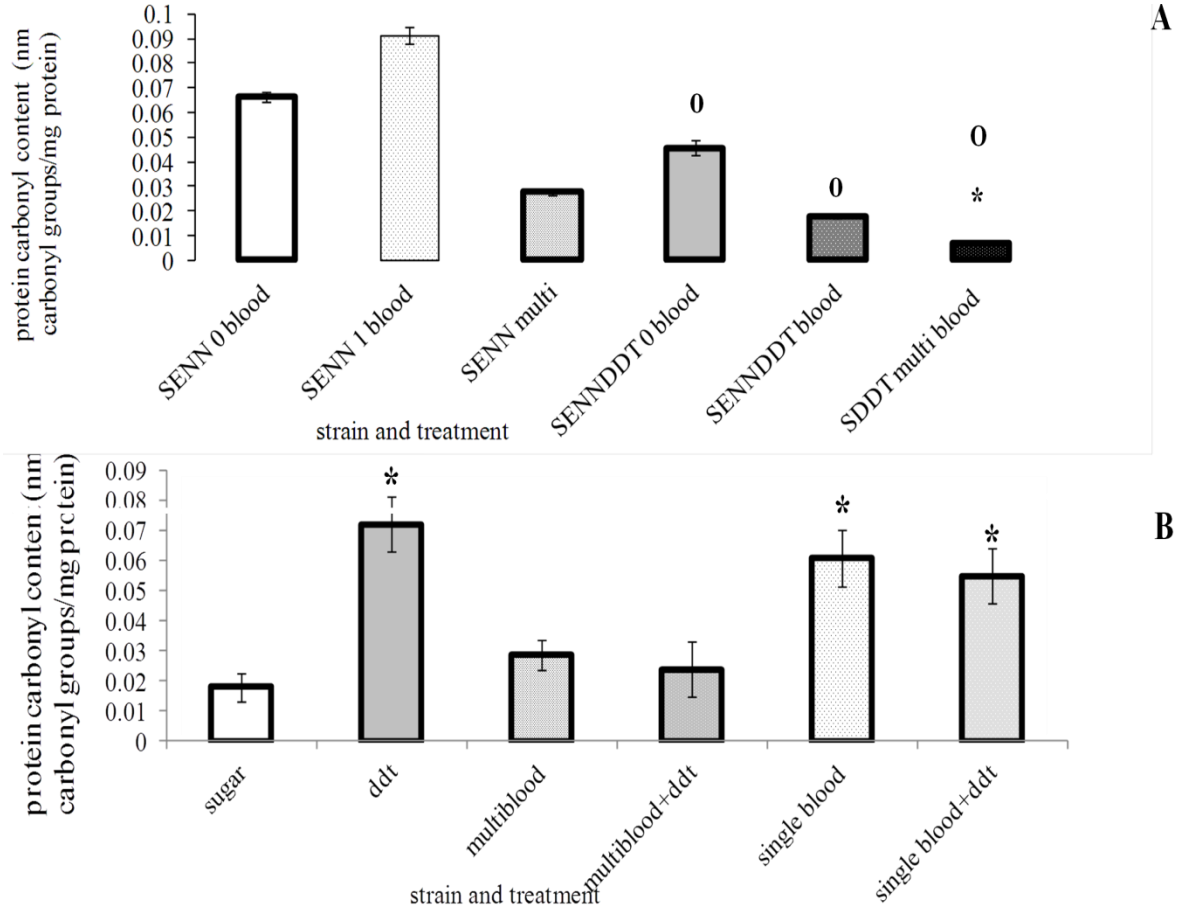


Figure 5.3.7: The effect of blood-feeding on the protein carbonyl content, a measure of oxidative stress, in *Anopheles arabiensis* SENN and SENN DDT. Block A represents the effect of blood-feeding alone on SENN and SENN DDT. Blood-feeding did not induce any significant changes in the carbonyl content of SENN. Multiple blood-meals resulted in a significant reduction in carbonyl content compared to unfed controls of the same age in SENN DDT (indicated by asterisk). SENN DDT always had a lower carbonyl content than equivalent SENN treatments. Block B represents the effect of blood-feeding on DDT-induced oxidative stress in SENN DDT. An asterisk denotes a significant increase in carbonyl content in comparison to the control. DDT treatment alone significantly increased carbonyl content. While a single blood-meal alone or in combination with DDT exposure resulted in higher protein carbonyl concentration, cohorts fed multiple blood-meals, with or without DDT exposure, did not differ significantly from a sugar fed control of the same age.

5.3.3.2 The modulation of the oxidative stress response to blood-feeding by oxidative stress enzymes

The previous results demonstrated that multiple blood-meals affected the oxidative stress profile, particularly in insecticide resistant females. This leads to the next question: how is this effect mediated? To answer this question, it is necessary to examine how the enzymes that reduce oxidative stress are affected by blood-feeding. Catalase is important single enzyme catalysing the scavenging of hydrogen peroxide (Preston *et al.*, 2001). Glutathione peroxidases are a class of enzymes that metabolise H_2O_2 and lipid hydroperoxides by catalysing the reduction of H_2O_2 (Arthur, 2000; Ahmad *et al*., 1989).These were the two enzyme systems examined. Figure 5.3.8 depicts the catalase activity of SENN and SENN DDT. Although SENN DDT females had a significantly higher level of catalase activity than their SENN counterparts (2 sample t-test: $p<0.05$; $t=-3.04$), this effect was lost later in life, where no significant differences were recorded between SENN and SENN DDT adults of 15 days of age, regardless of blood-feeding status (1-way ANOVA: $p=0.54$, $F=0.82$, $df= 5$). Blood-feeding did not result in significantly increased catalase activity in SENN (1-way ANOVA: $p=0.92$, $F=0.07$, $df=2$) or SENN DDT (1 way ANOVA: $p=0.75$, $F=0.30$, $df=2$).

Glutathione peroxidase is also an important class of enzymes involved in the oxidative stress response. Multiple blood-meals in SENN DDT significantly increased the activity of this enzyme class compared to sugar-fed controls (2 sample t-test: $p=0.01$; $t=-2.45$). This did not hold true for SENN, where multiple meals did not result in a significant increase in enzyme activity (1 way ANOVA: $p=0.95$, $F=0.8$, $df=2$) (Figure 5.3.9).

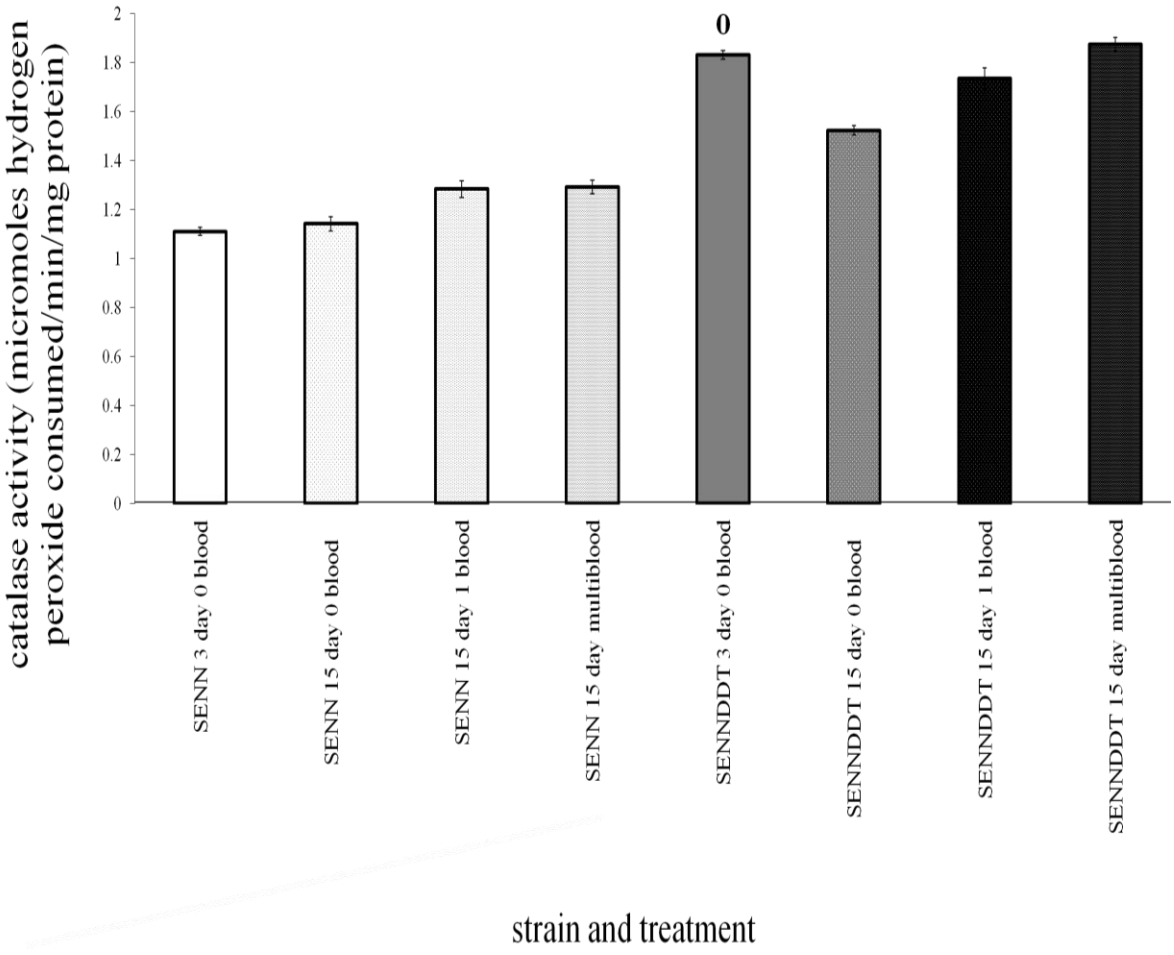


Figure 5.3.8: Variation in catalase activity of *Anopheles arabiensis* SENN and SENN DDT with age and blood-feeding treatment. At the age of 3 days, SENN DDT showed a significantly higher level of catalase activity than SENN (denoted by a circle). However, this effect was lost with age. For both SENN and SENN DDT there was no variation in catalase activity at the age of 15 days, regardless of blood-feeding treatment.

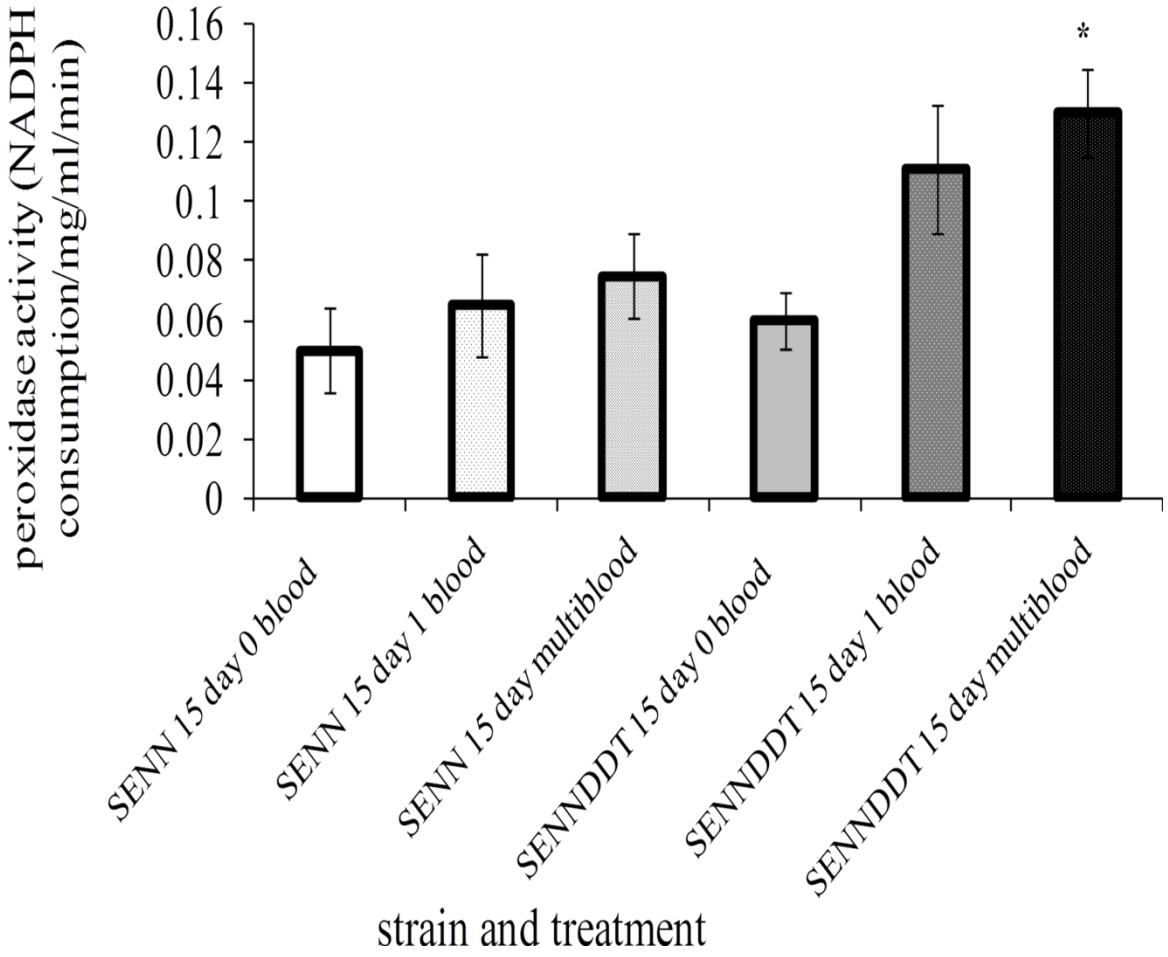


Figure 5.3.9: Variation in glutathione peroxidase activity of *Anopheles arabiensis* SENN and SENN DDT with blood-feeding treatment. There was no significant variation in glutathione peroxidase activity in SENN regardless of blood-feeding treatment. Multiple blood-meals induced a significant increase in enzyme activity in SENN DDT. For the sugar-fed and single blood-meal treatment there was no significant difference in peroxidase activity between SENN and SENN DDT cohorts of the same age.

5.3.4 Diet and the longevity of female *An. arabiensis*

Although insecticide resistance will play a crucial role in malaria vector capacity, longevity is also an important consideration. Factors that reduce longevity will play a critical role in the reduction of vector capacity, as the female needs to live beyond the extrinsic incubation period of the parasite to transmit the disease (Day, 1954).

5.3.4.1 Insulin and longevity

Human insulin is a serum component of blood and a hormone that the female is highly likely to come into contact with during her lifetime. It has previously been demonstrated that exogenous insulin reduces the lifespan of *An. stephensi* (Kang *et al.*, 2008) (Kang MA *et al*., 2008). This study was only performed on insecticide susceptible females. In this study, the effect of insulin was examined on both insecticide resistant and susceptible females. Insulin did not have a significant effect on the longevity of the insecticide resistant SENN DDT strain (Log rank test: $p=0.95$; $\chi^2=0.25$) (Figure 5.3.10B). As can be seen in figure 5.3.10A, however, insulin significantly reduced the longevity of their susceptible SENN counterparts (Log rank test: $p=0.01$, $\chi^2=15.52$).

5.3.4.2 Blood, mating and longevity

Figure 5.3.10 C represents the effect of blood-feeding on SENN longevity, while Figure 5.3.10 D represents the effect of blood-feeding on SENN DDT longevity. For SENN, BSA resulted in a significant decrease in longevity (Log rank test: $p=0.01$; $\chi^2=8.68$). Multiple blood-meals resulted in a significant increase in longevity in unmated SENN females (Log rank test: $p=0.02$, $\chi^2=5.29$), but not mated females (Log rank test: $p=0.14$, $\chi^2=2.22$). BSA did not have a significant effect on the longevity of SENN DDT females (Log rank test: $p=0.95$; $\chi^2=0.25$). Blood-feeding, however, did have a significant effect on the longevity of the strain. The lifespan of mated females was increased (Log rank test: $p<0.05$; $\chi^2=13.74$) as was that of unmated SENN DDT females (Log rank test: $p<0.05$; $\chi^2=11.21$).

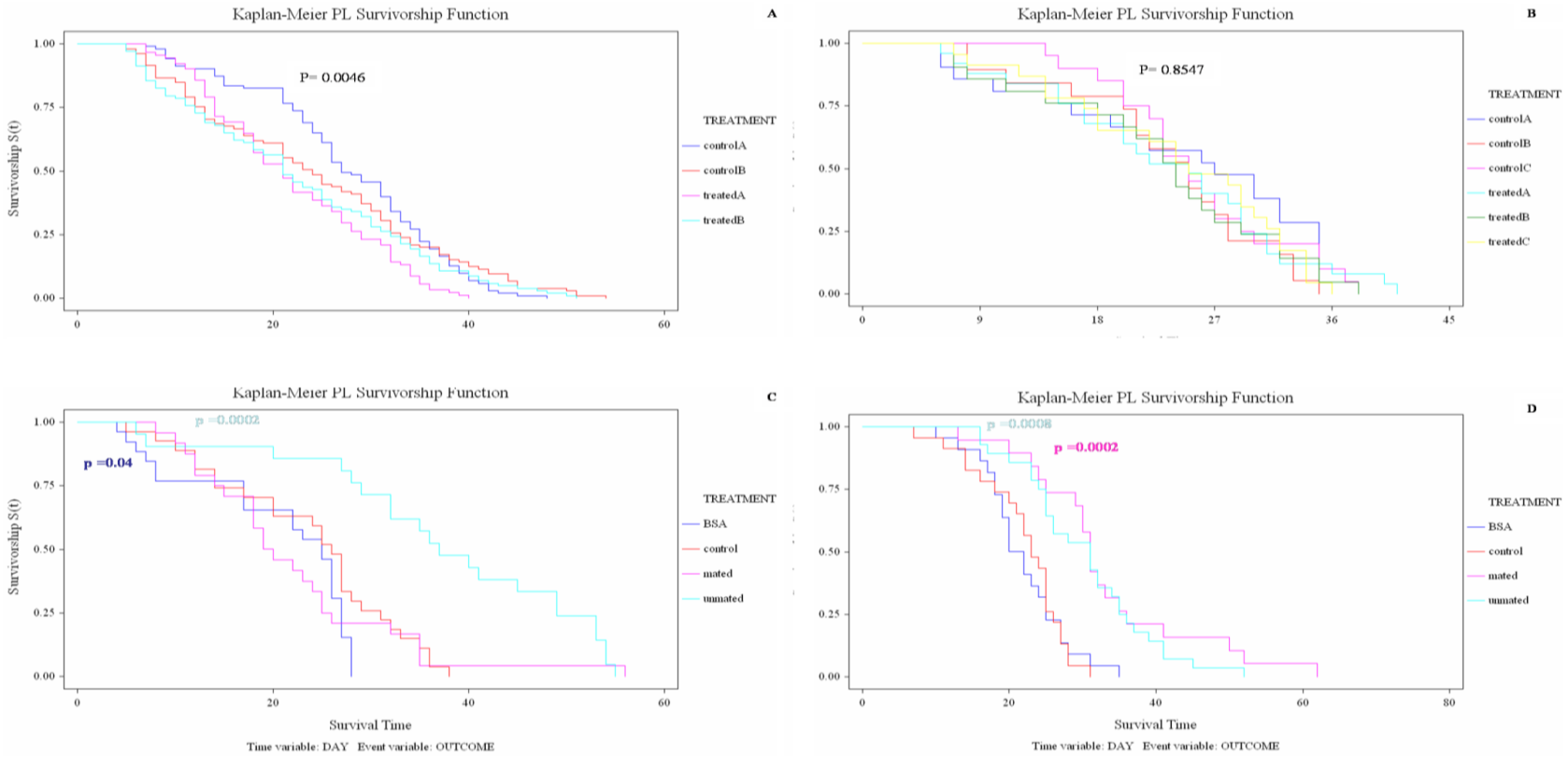


Figure 5.3.10: The effects of diet on the longevity of *Anopheles arabiensis* SENN and SENN DDT. Block A shows that active recombinant human insulin supplemented to SENN via sugar water significantly reduced the lifespan of SENN females (pink and turquoise lines- insulin treatment, dark blue and red lines-controls). This was not mirrored in SENN DDT (Block B), where insulin did not result in any sugnificant changes in longevity. Block C highlights the effect of blood and serum proteins on the longevity of SENN. BSA (dark blue line) significantly reduced longevity compared to the control (red line). Although blood-feeding significantly increased the longevity of unmated females (turquoise line), it did not increase the longevity of mated females (pink line). Again, the situation for SENN DDT is different (Block D). BSA did not have a significant effect on longevity. Blood-feeding, however, did result in a significant increase in longevity for both mated and unmated SENN DDT females.

5.4 Discussion

Of all the results presented in this study, the most significant is the effect of blood-feeding on the insecticide resistant phenotype. This is primarily due to its' potential operational impact, but also because it both complements and contrasts previous studies. In this study, a single blood-meal was administered at various stages of the mosquito's life. In previous studies that examined the effect of a single blood-meal, the study usually examined the resultant effect on young mosquitoes (Halliday and Feyereisen, 1987, Spillings *et al*., 2008). Although this study reinforces the fact that a single blood-meal in a young mosquito reduces insecticide sensitivity where insecticide resistance is already present, this was not the primary reason with providing females with single blood-meals. This study demonstrates that it is not a blood-meal administered at any age that makes a profound difference, but rather that the effect of multiple blood-meals is a cumulative one, and results from global physiological changes induced by multiple blood-meals.

It is worthwhile noting that for permethrin and deltamethrin a single blood-meal at the age of 11 days resulted in a significant reduction in mortality. Previous studies have stated that a single blood-meal at the age of 14 days does not affect insecticide resistance (Rajatileka *et al*., 2011), and it is usually assumed that female insecticide susceptibility would already have increased by this age. This age is significant as this is the age when the shortest intrinsic incubation period of the parasite is reached (Day, 1954). Although it is clear that the changes in the female due to multiple blood-meals, rather than a single blood-meal, are at the root of the blood-feeding response, it should be noted that the effect of a single meal is not to be ignored. In the oldest cohorts (18 and 21 days) a single blood-meal had curious and contrasting effects on permethrin and deltamethrin induced mortality. For the permethrin treated group, it resulted in a significant increase in mortality, and was therefore a disadvantage. For deltamethrin treated females, however, it proved advantageous, and resulted in significantly reduced mortality. It is of concern that a single blood-meal could still elicit a positive effect in mosquitoes of that age.

This brings us to the single most important result of this study: multiple blood-meals result in significantly lower DDT, permethrin and deltamethrin induced mortality with age. In the case of DDT and permethrin, it sustains the phenotype until the female is 21 days old. The major

implication of this finding is that an age-induced increase in insecticide sensitivity is not a given. The operational implications of this will be discussed, but as this finding is in contrast to previous studies, it is important to validate why this may be.

The first reason for this may be a result of sample size. In previous studies (Rajatileka *et al*., 2011), sample sizes ranged between 83 and 121. While this study was being performed, it was observed that although a trend would be observed, it only became significant when larger samples sizes (500 individuals and more) were used. Previous non-significant results may therefore have been due to small sample sizes.

The second reason, and perhaps a more important reason, for the results observed is the unique nature of the resistant laboratory strain, SENN DDT. This strain is homozygous resistant for the *kdr* east (L1014F) mutation, and has elevated cytochrome P450, GST and general esterase activity. In the study that teased out the effect of age and blood-feeding on insecticide resistant mosquitoes (*Ae. aegypti* and *An. gambiae*) with known resistance mechanisms, none of the strains had the same combination of mechanisms. It is important to note that in the RSP strain, which had elevated GST activity, elevated P450 activity as well as the *kdr* L1014 S mutation, a similar pattern was noted. Even with a small sample size (n=102), a significant reduction in DDT-induced mortality was observed in 14 day old individuals fed a blood-meal compared to 3 day old females that were sugar fed (Rajatileka *et al*., 2011).

The results from the multiple blood-feeding cohort have two major impacts. The first is that, with the right combination of resistance mechanisms, senescence does not automatically result in increased insecticide sensitivity. It has been suggested that ageing may partially restore vector control efforts (Chouaibou *et al*., 2012, Jones *et al*., 2012a). This study, performed on blood-fed wild material, only took *kdr* status into account and did not consider metabolic involvement. Furthermore, a 2011 study suggested that continuous low levels of insecticide exposure via fogging would kill older, more susceptible females without placing a selection pressure on younger females (Glunt *et al*., 2011). The results of this study suggest that this is not necessarily the case. More importantly, it shows the importance of considering environmental factors when tailoring age-targeted vector interventions. It is also important to note that the very same study that suggested using continuous low-level fogging reported that a single blood-meal reduced pyrethroid toxicity (Glunt *et al*., 2011).

The second major impact is that the results of this study may help answer the question of whether *kdr* as a single point mutation can confer a resistance phenotype. As discussed in Chapter 1, the *kdr* mutation is a single amino acid change from leucine to either serine or phenylalanine in the *para* voltage gated sodium channel (VGSC). This mutation makes the VGSC least sensitive to inactivation by DDT, then permethrin and most sensitive to deltamethrin inactivation (Reimer *et al*., 2008). The biology of the L1014F mutation has been described. It is a primary resistance mutation in Domain II of the sodium channel (Soderlund and Knipple, 2003). The L1014F mutation shifts the channel to a more positive potential, where closed state inactivation is promoted. Therefore, with the L1014F mutation, 70-80% of the channels never open, and DDT and pyrethroids preferentially bind to open channels (Davies *et al*., 2007). DDT is the least effective at binding the sodium channel, permethrin more effective and deltamethrin the most effective at binding the channel (Davies *et al*., 2007). This explains the order in which resistance is associated with *kdr*-mediated resistance.

As the L1014F mutation slows down the penetration of the insecticide into electrically active cells, rather than preventing them from entering the cell, the lipid-soluble xenobiotics will still be present at toxic levels in the cell. They would therefore still need to be detoxified and excreted. Brooke (2008) and Donelly *et al.* (2009) have suggested that "other factors" are involved in *kdr*-mediated resistance, and this study suggests that these factors are metabolic detoxification mechanisms (Brooke, 2008, Donnelly *et al*., 2009).

Firstly, from Chapter 4, it is known that all insecticides induce oxidative stress. Therefore, insecticides that have not acted on the nervous system must still be metabolised or they will induce death by oxidative damage. From this study it is clear that multiple blood-meals maintain the resistance best for DDT, then permethrin and finally deltamethrin. For all three insecticides, including the two most closely associated with *kdr*, in the absence of blood-feeding, susceptibility to insecticides increases with age. Maintenance of detoxification enzyme activity (as will be discussed later) in conjunction with the *kdr* mutation is the best means of maintaining the insecticide resistant phenotype. Therefore, it is highly likely that *kdr*-mediated resistance functions best in conjunction with metabolic detoxification mechanisms.

It is a notable exception to the multiple blood-meal effect that the malathion resistant phenotype is not as strongly affected by blood-feeding. This is not an unexplainable event. It

has been noted that blood-feeding has a variety of effects on insecticide resistant phenotype, increasing resistance to the pyrethroid fenvalerate, decreases the resistance to carbamate, and does not affect resistance to aldrin (Halliday and Feyereisen, 1987). Therefore, the effect of blood-feeding is not universal and the classes of insecticides that it affects give significant clues about the underlying biological mechanisms. Furthermore, it has previously been noted that with a single blood-meal, the blood-feeding did not have an effect on malathion resistance (Eliason *et al*., 1990). Whether this is an effect restricted solely to malathion, or whether it is common to all organophosphates needs to be investigated.

Insulin is a host blood component that mediates various effects in haematophagous insects (Kang *et al*., 2008, Riehle and Brown, 1999). This study provides the first data of the effect of exogenous insulin on the insecticide resistant phenotype. Firstly, it proves that the hormonal effect of insulin can manipulate the response to DDT, rather than an increase in protein supplementation, as denatured insulin did not affect the resistance phenotype. The reduction of DDT-induced mortality is however very short-lived, and the effect is lost after 7 days. This could possibly be due to the means of administration of the insulin. Exogenous insulin would not normally be taken up during a sugar meal but rather during a blood-meal. A sugar meal would be stored in the diverticulum until it is digested in the midgut. This may explain the loss of activity after a few days. Insulin is a small (5.8 kDa), labile peptide (Voet and Voet, 1995) that is easily digested and it is for this reason that insulin is not taken orally by humans (Carino and Mathiowitz, 1999, Kuo *et al*., 1991). The mosquito midgut contains insulin degrading enzymes (White *et al*., 1987), in addition to being highly basic (Clements, 2000), all of which would result in inactivation of the hormone. As a mosquito would ingest insulin with the blood-meal and would be digested relatively rapidly, and it is therefore unlikely that the hormone ingested in this way will have any meaningful effect on the DDT resistance phenotype. Although the result is interesting, it is unlikely to have operational effects.

Although the effect of blood-feeding on phenotype is interesting in itself, it leads to the question of how this is biologically mediated. Although Halliday and Feyereisen (1986) elegantly demonstrated how the effect was not mediated, they could not give an explanation of how blood-feeding reduced insecticide toxicity.

In the study of blood-feeding on *An. funestus*, Spillings *et al.* (2008) hypothesized that the change in pyrethroid detoxification in resistant *An. funestus* was due to increased cytochrome P450 activity. This conclusion was based on two studies; one showed that increased aldrin epoxidation in *Culex pipiens* after a blood-meal (Baldridge and Feyereisen, 1986) and a microarray analysis of changes in transcript abundance after a blood-meal in *Ae. aegypti* (Sanders *et al*., 2003). The explanation sounds feasible, but subsequent studies, including this study, suggests that the answer is less intuitive.

A microarray analysis of pyrethroid resistant *An. arabiensis* from Cameroon showed that CyP6P3 and a variety of other cytochrome P450 transcripts were significantly downregulated after a blood-meal (Muller *et al*., 2008). This was again confirmed in both *An. gambiae* and *An. funestus* by microarray analysis (Spillings, 2012). The present study is the first to add biochemical evidence to these transcriptomic findings. Importantly, this finding was only observed in insecticide resistant mosquitoes. Neither Baldridge and Feyereisen (1986) nor Sanders (2003) detail the resistance status of their insects. Both Muller *et al*. (2008) and Spillings *et al*., (2008) highlight the fact that GSTs are upregulated in resistant blood-feeding insects, implicating this class of enzymes in the response. Interestingly, blood-feeding did not have a neat effect on esterase activity, which was only significantly increased by multiple blood-meals after the age of 15 days and beyond. This may explain the behaviour of the malathion resistant phenotype.

A 2008 study highlights the fact that a strong anti-oxidant defence system was mounted in response to pyrethroid intoxication (Muller *et al*., 2008). Although cytochrome P450s are the major enzymes involved in pyrethroid resistance, GSTs mediate pyrethroid resistance by reducing oxidative stress-induced oxidative cytotoxicity (Vontas *et al*., 2001). A study in *Ae. aegypti* demonstrated that blood-meals reduce midgut oxidative burden (Oliveira *et al*., 2011). Therefore, it may be that a female mosquito's mechanism of coping with blood-meal induced oxidative stress has the downstream effect of reducing insecticide toxicity as well. The response observed in mosquitoes that have taken multiple blood-meals may be because with each blood-meal the female becomes primed for the oxidative burden, and subsequently lowers ROS-induced cytotoxicity.

The results of this study suggest that the hypothesis described by Muller *et al.* (2008) is correct and that defence against oxidative stress underlies the response to multiple blood-

meals. When examining the enzymes involved in the multiple blood-meal induced reduction of oxidative stress, it appears that the effect is mediated by the glutathione peroxidase activity, rather than catalase activity. As insects lack selenium-dependent glutathione peroxidases most peroxidase activity is mediated by GSTs (Simmons *et al*., 1989a, Simmons *et al*., 1989b). The multiple blood-meal induced GST activity coincides with increased peroxidase activity. All these results indicate that GST activity may be at the root of blood-meal induced resistance, which may potentially explain the variation in responses to different insecticides after one or multiple meals.

An obvious question is why is the effect of multiple blood-meals on insecticide sensitivity only observed in insecticide resistant mosquitoes? From Chapter 4, we have learned that insecticide resistant mosquitoes have an enhanced capacity for coping with oxidative stress. In this study, we see further practical applications of this adaptation. While it is clear that multiple blood-meals result in a lower nett oxidative damage in the insecticide resistant female, this is not the case for the susceptible mosquitoes, where multiple blood-meals do not induce such a dramatic reduction of oxidative stress. The reduction of P450 activity and the increase in GST activity suggests that with multiple blood-meals, the system of the resistant mosquito responds to minimise oxidative stress. This response was probably initially directed only at coping with the reduction of the oxidative burden induced by blood-feeding, as it has been demonstrated in *Ae. aegypti* that a blood-meal lowers oxidative stress in the midgut (Oliveira *et al*., 2011). Increased defence against the oxidative burden of insecticides as well as increased defence against the oxidative burden of multiple blood-meals in the insecticide resistant mosquito most likely evolved in a synergistic manner. Insecticide susceptible mosquitoes that do not have increased oxidative stress defence capacities are unlikely to mount as strong an antioxidant response to a blood-meal and therefore a blood-meal is unlikely to have the strong downstream response of defence against insecticide-induced oxidative cytotoxicity. There also appears to be no cumulative increase in oxidative defence with multiple blood-meals in the insecticide susceptible female, and therefore the "priming" of the system to cope with repeated ROS onslaught does not appear to happen in these individuals. This may underlie the reason why blood does not offer an advantage in terms of insecticide sensitivity in these individuals.

Factors affecting longevity in anophelines is a remarkably unexplored topic. At the time of writing, only14 papers were published on the subject. As small changes in longevity have a

large impact on disease transmission (Garrett-Jones and Shidrawi, 1969), this study addressed some of the environmental factors affecting longevity. As with the experiment about insulin and insecticide resistance, this study builds on work about insulin and longevity in *An. stephensi*. The 2008 study, the only one to date on the subject, proposed the hypothesis that exogenous insulin shortens the lifespan of the mosquito, and that as hyperinsulinaemia is common in malaria patients, that this may inadvertently lead to the shortening of the lifespan of mosquitoes feeding on malaria patients (Kang *et al*., 2008). Our results do not fit neatly with those results. As Kang *et al.*,(2008) demonstrated that insulin supplementation via both blood and sucrose had an effect on longevity, this study chose to focus only on insulin supplementation via sucrose.

From the insecticide resistance study we have learned that insulin administration via this route could have an effect on phenotype. What made this study different to the Kang *et al.* (2008) study was that insecticide resistant phenotype was taken into account. Kang *et al.*, (2008) used "*Anopheles stephensi* Liston (Wild Type Indian Strain)" which would, presumably, be insecticide susceptible. This proved to be a very important factor. While the results of the insecticide susceptible SENN strain mirrored those in *An. stephensi*, the insecticide resistant SENN DDT strain was in contrast to the previous results, with insulin not having a significant effect on longevity. The hypothesis for the effect of insulin on longevity was that insulin-induced oxidative stress reduced longevity. From the study described in Chapter 4 as well as the present study, it is clear that insecticide susceptible strains do not cope as well with oxidative stress as their resistant counterparts. This may be the reason why the effect of insulin is negated in the SENN DDT strain. Therefore, if insulin consumption by mosquitoes is to have an effect on populations, it is most likely that it will be limited to insecticide susceptible populations.

The longevity study, yet again, shows the importance of oxidative stress in the life of anophelines. Similar to the insulin experiment, consumption of the large BSA monomer (66.5 kDa), significantly decreased the lifespan of insecticide susceptible, but not resistant, females. The reasoning for this is the same as for insulin consumption, but this result is interesting in terms of the theory of caloric restriction and ageing. In studies ranging from *Drosophila* to mammals, the debate about whether caloric restriction affects lifespan is continuous (Barja, 2002, Masoro, 2005, Sohal *et al*., 2002). In contrast to a study in rats (Caro *et al*., 2009), this study suggests that additional dietary amino acids induce oxidative

stress in mosquitoes, but that the effect is limited to insecticide susceptible mosquitoes that cannot cope with the oxidative burden.

Caloric restriction has been demonstrated to increase longevity (Roth and Polotsky, 2012), although the efficacy of this treatment is a subject of much debate for invertebrates (Mendelsohn and Larrick, 2012). It is therefore interesting that a diet of sugar and blood, with an increased caloric load compared to a sugar only diet would have such a positive effect on mosquito longevity. This appears to be related to reproductive function, rather than lining up with caloric and oxidative stress theories of ageing. A previous study also showed that in *An. gambiae* sugar and blood was the best dietary combination to ensure extended longevity (Gary and Foster, 2001). In reference to this study, it is unsurprising that a diet of blood alone was unable to result in long-lived mosquitoes, as unlike the *Glossinidae*, mosquitoes cannot sustain flight on blood, and therefore need sugar as energy source (Lane and Crosskey, 1993). The results of the current study suggests that that blood and sugar-feeding is the best dietary scheme for *An. arabiensis*, and that reproduction may play a role in longevity.

Only one study, conducted in *An. gambiae*, examined the effect of mating on subsequent longevity (Dao *et al*., 2010). This is in contrast to a multitude of studies in *Drosophila* where mating is known to come at a price. Male reproductive proteins shorten the lifespan of the female (Fiumera *et al.*, 2006; Wigby and Chapman, 2005). Whether or not this is true for anophelines needs to be investigated. What we learn from this study is that multiple blood-meals increased longevity in *An. arabiensis*, but that reproductive status plays a role. For insecticide resistant SENN DDT, multiple blood-meals increased longevity regardless of mating status, while for their susceptible counterparts, it only increased longevity in unmated females. The underlying mechanism for this is unknown but it does suggest a mating cost for the susceptible SENN strain. It may be argued that old unmated females are of no medical importance, as they are unlikely to occur in nature, and they are not passing on genetic material. This, however, just highlights the advantage that multiple blood-meals have in insecticide resistant populations.

A final consideration about blood and adult longevity has to do with sugar feeding. In nature, *An. gambiae* may forego sugar feeding in favour of blood feeding (Gary and Foster, 2001). This may probably be because anophelines are not as good as “meal switching” as *Aedes* species, and therefore need to be more ready for a blood-meal. *Anopheles gambiae* is more

anthropophilic and less opportunistic than its sibling species *An. arabiensis*, and it is not known whether *An. arabiensis* will forego sugar feeding in the wild. This study suggests not, as sugar and blood appears to be the best dietary regime for the species. This is, however, the case for an established laboratory strain, and this would require confirmation in wild mosquitoes.

This study has presented numerous data about the effects of blood-feeding on the insecticide resistant phenotype, life history and biology of *An. arabiensis*. It has shown that multiple blood-meals augment the DDT, permethrin and deltamethrin resistant phenotypes, and maintains the DDT and permethrin resistance phenotype. This effect is primarily mediated by the modulation of oxidative stress. Unlike suggestions made in previous studies, this affect appears to be mediated by GSTs, which are primed to result in a reduced nett oxidative burden through increased peroxidase activity, but also through sustained xenobiotic detoxification by continuously elevated transferase activity. Previous studies have demonstrated that GSTs are capable of sustaining a resistance phenotype. These include fenopropathrin resistance in the red citrus mite *Panonychus citri* (Liao *et al*., 2013), deltamethrin resistance mediated by GSTe7 and GSTe2 in *Aedes aegypti* (Lumjuan *et al*., 2011) and decamethrin resistance in the red flour beetle *Tenebrio molitor* (Kostaropoulos *et al*., 2001). This effect does not occur in insecticide susceptible females, which may explain why the effect of multiple blood-meals on insecticide toxicity is restricted to insecticide resistant mosquitoes. Due to the oxidative stress response exogenous dietary stressants that would result in ROS accumulation reduce the longevity of insecticide susceptible, but not resistant, females. Multiple blood-meals increase the longevity of *An. arabiensis*, but for insecticide susceptible females there appears to be a mating cost. The data presented in this study suggest that increasing insecticide sensitivity in the senescent mosquito is not a given, and that special consideration needs to be taken before attempting to tailor age-targeted vector control efforts. Most importantly, ageing does not necessarily restore vector control efforts. This study also highlights key biological differences between insecticide resistant and susceptible mosquitoes and shows an important practical application of the findings from an earlier-described study about the oxidative stress capacity of resistant mosquitoes.

Chapter 6: Discussion and Conclusion

The overall finding of this study was that each of the stressors examined did affect, the expression of insecticide resistance phenotypes and life history traits, either positively or negatively.

The last two of the original five objectives have not been fully addressed in their respective chapters, as they can only be answered by making a holistic examination of the subject matter, which could only be done at the end of the study. The two objectives in question concern the interplay between metabolic and target site resistance mechanisms, and the interplay between the stress response systems and the metabolic detoxification systems.

One of the primary conclusions of this study is that the maintenance of the *kdr* phenotype is most effective when combined with a metabolic detoxification system. The *kdr* mutation is possibly the best target site mutation to use as an example, as unlike the *rdl* and *ace-1* mutations, it does not render the target site completely insensitive, but rather reduces the bioavailability of the insecticide by having a high proportion of the sodium channels being in a closed conformation, instead of the open conformation preferential for insecticide binding (Davies *et al*., 2007). Insecticide can therefore still accumulate in the system even though it has not reached its' target site. From chapter 4 it is apparent that insecticides induce oxidative stress, and therefore still need to be removed from the system. An enhanced capacity for metabolic detoxification is therefore the perfect complement for the optimal expression of resistance. These results support those presented in a recent publication demonstrating that metabolic and target site resistance can combine to form a strong DDT resistance phenotype (Mitchell *et al*., 2014).

Chapter 5 presents results which demonstrated that maintenance of the efficacy of *kdr* phenotypes in the ageing mosquito occurred due to elevated GST activity induced by multiple blood meals. In a female mosquito deprived of blood for her entire life, insecticide susceptibility increases with age and the female becomes more prone to insecticide intoxication (Lines and Nassor, 1991). Multiple blood meals augment and maintain insecticide resistance in older mosquitoes, and this effect is most marked in phenotypes closely associated with *kdr*-mediated resistance (DDT>permethrin>delatamethrin). The fact that this effect is absent in the malathion resistance phenotype, which is completely

independent of the *kdr* mutation, and solely metabolically mediated in the strain examined, is one of the most compelling pieces of evidence. Whether this is true for all target site mutations, which could not be examined as they were not present in the resistant strains under examination, needs to be investigated.

Closely related to the knowledge that *kdr* does not act in isolation is the concept that metabolic detoxification systems do not act in isolation either. The concept of the existence of a "pyrethrome", a system of interacting proteins involved in the metabolic detoxification of pyrethroids (Ismail *et al*, 2013) suggests that metabolic enzymes working in concert to detoxify a xenobiotic compound. The need for the detoxification of secondary and tertiary metabolites of the primary insecticide (Yu, 2008) is strongly suggestive of an integrated system of detoxification. Furthermore, despite the lack of functional data, the ABC transporter family does present the best potential orthologue for the active transporters that constitute the Phase III detoxification systems in mammals (Dermauw and Van Leeuwen, 2013). This would also mean that detoxification systems in insects may be more similar to mammalian detoxification systems than previously imagined.

Given the evidence for target site-metabolic interactions as well as inter-metabolic detoxification interactions, it is necessary to examine the next level of potential interactions between metabolic detoxification systems and other biological systems. In vector biology, metabolic detoxification and insecticide resistance is often considered in isolation. When insecticide resistance mechanisms are considered within a broader biological context, it is usually within the scope of fitness costs. There are, however, a few studies on the effect of insecticide resistance on the behaviour of an organism (Kliot and Ghanim, 2012, Malima *et al*., 2009). The fact that stimuli and environmental stressors such as larval nutrition can modulate the insecticide resistance phenotype is an indication that different systems interact and manipulate the metabolic detoxification system.

Although regularly mentioned as a factor in pyrethroid resistance, not much information is available concerning the role of oxidative stress defence in insecticide resistance since first being described by Vontas *et al*, (2001). In the present study the association between insecticide resistance and increased oxidative stress capacity highlights the importance of that finding. The fact that the defence against oxidative stress is most marked in the *An. funestus* FUMOZ R strain where insecticide resistance is primarily monofactorial is most telling. It

suggests that increased oxidative stress defence is required to cope with a highly active P450 system in this strain, leading to high systemic levels of ROS (Siraki *et al*., 2002, Zhang *et al*., 2011). This potential co-evolution indicates that metabolic detoxification systems have an effect on other biological systems.

The blood feeding results presented here also provided a good example of interacting systems. At the end of their exhaustive study to understand "Why DDT toxicity decreased after a blood meal" Halliday and Feyereisen (1987) concluded that they could not establish why this effect occurred. The present study suggests two possible answers, which more than likely represent a single answer due to systems interaction. In chapter 4, it was demonstrated that insecticide resistant *An. arabiensis* and *An. funestus* had an elevated capacity for coping with oxidative stress. Secondly, a hypothesis was presented that the already elevated oxidative stress capacity of these resistant females was further heightened by the response to the oxidative stress induced by blood feeding. This extremely heightened defence system reduced the ROS-induced insecticide toxicity, accounting for the reduced toxicity of the insecticide after a blood meal. This hypothesis also accounted for the observations of Spillings *et al*. (2008) that blood-meal induced reduction of insecticide toxicity was restricted to females possessing the resistant phenotype. The reduction of cumulative oxidative stress as a contributing factor to bloodmeal-induced reduction of insecticide toxicity is by no means the only possible explanation of the effect of multiple bloodmeals on insecticide resistance. The bloodmeal also serves as a heatshock to the system of the female, and results in the production of heat shock proteins as a cytoprotective measure (Benoit *et al*., 2011). It has been demonstrated that thermal stress can induce cross tolerance to insecticides and that the presence of insecticides can result in the production of the inducible chaperone protein heat shock protein 70 (Patil *et al*., 1996). Therefore, the heatshock induced by a bloodmeal, particularly the continued shock of multiple meals may contribute to the resistance phenotype. This requires further investigation, and would also represent an example of multi-systems cross-talk.

Several studies show evidence of the increasing susceptibility of ageing adult females to insecticides (Rowland and Hemingway, 1987, Lines and Nassor, 1991; Rajatileka *et al*., 2011, Chouiabo *et al*, 2012). As a consequence, age targeted vector interventions have been suggested (Glunt *et al*., 2011) and the use of late-acting insecticides, based on the concept of increased susceptibility in older mosquitoes, has been suggested as an "evolution proof"

intervention (Read *et al*., 2009). However, the data presented here showing that older females that have taken multiple blood meals are not as insecticide susceptible as expected is a major consideration for innovative vector control efforts designed to exploit age variation in insecticide susceptibility. These data also suggest that the toxicity levels of future insecticide products could possibly be enhanced by using an oxidative stress defence system synergist such as ATZ in addition to other enzyme synergists such as PBO. The aforementioned examples show that there are several cross-system interactions involved in the mediation of insecticide resistance. The major implications of these findings are that they highlight the complexity of the mechanisms governing insecticide resistance. The FUMOZ R strain, a pyrethroid selected strain from Mozambique, has been demonstrated to have a pyrethroid resistance phenotype conferred by the overexpression and elevated enzyme activity of the P450 CYP6P9 and its' duplicate CYP6P13/CYP6P9b (Wondji *et al*., 2009; Matambo *et al*., 2010). The fact that the GST synergist haematin significantly increased pyrethroid-induced mortality in this strain suggests that the elevated GST activity observed in this strain (Brooke *et al*., 2001) also plays a role in pyrethroid resistance. Furthermore, the fact that the catalase synergist ATZ synergises pyrethroid resistance almost as well as PBO also casts doubt on the monofactorial nature of insecticide resistance in this strain. Rather than being a metabolic waste, the elevated GST activity, as well as the concomitant increased oxidative stress defence capacity, represents an important component of the pyrethrome of FUMOZ R. Although no GSTs were identified in an earlier mapping of a quantitative trait locus associated with pyrethroid resistance (Wondji *et al*., 2007), upregulated GSTs were noted in a microarray analysis of the strain (Christian *et al*., 2011), adding further credence to the importance of GSTs in pyrethroid resistance in *An. funestus*.

Environmental stress plays a critical role in the biology of the mosquito, and data presented here highlight the importance of the larval environment in shaping the life history of adults. Previous studies have shown that larval development, adult size and reproductive capacity is dictated by larval environment (Dmitriew and Rowe, 2011, Joy, 2010). Most considerations about the effect of larval environment on insecticide resistance in adults relates to insecticide selection pressure at the larval stage via runoff into breeding sites from agricultural insecticide use (Mzilahowa *et al*., 2008, Whiting *et al*., 2014). This study shows that other factors in the larval environment also shape the adult resistance phenotype, notably larval nutrition and temperature conditions, and raises the possibility that additional factors in the

larval milieu may also modulate adult response to xenobiotics. Potential future studies could address the effect of other sources of environmental stress on the life history and insecticide resistance phenotype of malaria vectors, particularly *An. funestus*. These include thermal stress and environmental pollutants. As warnings have already been raised that temperature affects the efficacy of insecticides (Glunt *et al*., 2013) and that insecticide resistant and susceptible mosquitoes have different capacities for thermal tolerance (Swain *et al*., 2008), thermal stress is an important environmental stress to consider.

The study of larval environmental stressors also provides another interesting example of multiple system interactions as well the interplay between target-site and metabolic resistance. In the SENN DDT strain, DDT resistance is primarily mediated by the *kdr* mutation (Nardini *et al*., 2012), yet insufficient larval nutrition significantly reduced the resistance capacity of adults in a manner independent of the effects of vigour tolerance (chapter 3, figure 3.3.5). As the *kdr* genotype is presumably unaffected by larval nutrition, unlike metabolic detoxification systems which are suppressed, these data suggest an interaction between target site and metabolic resistance whereby construction of resistance phenotypes cannot be fully achieved by *kdr* mediated reduced target site sensitivity alone.

The second factor to be highlighted is that the issue of longevity in mosquitoes is an extremely complex matter. *Caenorhabditis elegans* and *D. melanogaster* are used as invertebrate model organisms for a number of fields including biogerontology. In this study, using techniques derived from *Drosophila* studies worked very well for some investigations, particularly for the study of oxidative stress (e.g. Gupta *et al*., 2007, Gupta *et al*., 2005). However, distribution and lifestyle differences between the two organisms complicate the direct transposition of studies of the environmental stresses examined in this study between these two organisms. An example of this is the manner in which oxidative stress affects longevity in *Drosophila* in contrast to the observations made on anophelines in this study.

Drosophila melanogaster presents a neater model for the free radical theory of ageing (increased accumulated oxidative damage underlies senescence) than does *C. elegans* (Clancy and Birdsall, 2013, Van Raamsdonk and Hekimi, 2010). It would be simple to assume that the lessons learned from *Drosophila* can be applied to mosquitoes. A recent publication suggests that permethrin resistant *An. gambiae* have reduced metabolic rates and higher ROS production, and further suggests that increased oxidative burden results in

insecticide resistant females being shorter lived (Otali *et al*., 2014). The data presented in this study are different from the aforementioned study and present a more complex and integrated view of longevity in *Anopheles* mosquitoes.

Like the Otali *et al*., (2014) study, the present study found that resistant *An. arabiensis* tend to be shorter lived than their susceptible counterparts. As detailed in chapter 3, apparent developmental differences may be related to inadvertent selection for a late-hatch phenotype in the insecticide resistant strain (Kaiser *et al*., 2010). Yet the longevity studies of *An. arabiensis* are in conflict with the *Drosophila* studies. From the work performed in this study, insecticide resistant *An. arabiensis* exhibit enhanced oxidative stress defence, and consequently have lower systemic levels of ROS.

Why then is lifespan reduced in insecticide resistant *An. arabiensis* adults? There are two potential answers to this question. The increased amount of metabolic detoxification activity suggests that insecticide resistant adults (chapter 3, table 3.3.1) may be more metabolically active than their susceptible counterparts. Furthermore, as the resistant strain (SENN DDT) is fixed for the *kdr* mutation, the compromised neural activity may induce a yet unidentified fitness cost. Both of these factors require further study. When coupled with a contrasting potential longevity study in *An. funestus*, which has no target site resistance and shows no appreciable fitness costs due to insecticide resistance, the factors governing longevity in anophelines may prove to be a fascinating study. It would be particularly interesting to see whether mosquitoes generally behave more like *Drosophila* or *C. elegans*, but from the evidence in this study, they do not fit the *Drosophila* model well.

At the beginning of this study a defence of the use of laboratory strains was given. Although all laboratory findings must ultimately be validated in wild mosquitoes, this study emphasises the importance and usefulness of laboratory strains for mosquito research. From a purely practical viewpoint, this study demonstrates insights that can be gained when examining laboratory strains due to the ease of availability of large numbers of test material, as well as having well-characterised underlying mechanisms of insecticide resistance. Where wild mosquito samples were available, this study showed that the results observed in laboratory strains were confirmed in wild mosquitoes, and in the case of catalase activity, the wild mosquitoes exceeded their laboratory counterparts (chapter 4, figure 4.3.5B). This highlights the fact that certain characteristics of laboratory strains can be predictive of characteristics

found in the field. Furthermore, the work in chapter 3 may have implications for field studies finding that maize pollen enhanced the development of *An. arabiensis* (Ye-Ebiyo *et al*., 2000).

What does need to be clarified, however, is which characteristics of laboratory strains are most conserved in field populations. Although this has previously been attempted in *An. gambiae* (Huho *et al*., 2007), the results were not conclusive. A future study could test the hypothesis postulated in chapter 2, that the predictive success of a characteristic is related to the metabolic cost. According to this hypothesis, traits with a high energetic cost (reproduction/immunity) would be more amplified in laboratory strains, which would have higher energetic reserves, and less predictive of field conditions than factors with a lower energetic cost (temperature tolerance).

A factor apparent from this study is that a better understanding of the fitness costs of *kdr* as well as other target site mutations in *Anopheles* mosquitoes is needed. Fitness costs of various aspects of insecticide resistance are well characterised in culicine mosquitoes (Rivero *et al*., 2011, Rivero *et al*., 2010) yet less information on anophelines is available (Kliot and Ghanim, 2012). This is particularly true for target site resistance. While *kdr* proved to be a complicating factor when examining the effect of multiple blood meals on resistance (chapter 5, figure 5.3.1) and resulted in weaker correlations in the oxidative stress studies (chapter 4, figure 4.3.6), the reality is that *kdr* in particular is usually found in combination with metabolic resistance (Awolola *et al*., 2009, Chandre *et al*., 1999, Zhong *et al*., 2013). This means that examining the effects of combined metabolic and target site resistance is crucial. The SENN DDT laboratory strain is an excellent candidate for such combined studies as all three major metabolic detoxification enzyme systems are upregulated and occur in tandem with *kdr*.

The concept of the pyrethrome, an interacting network of molecules to metabolise pyrethroids (Ismail *et al*., 2013), is worth pursuing. Investigations on how different biological systems interact with each other in the context of the internal biological milieu as well as how these interactions are affected by their external environment will provide valuable insight into the broader biological picture of these medically important insects. It is vital that we consider insecticide resistance in the context of an "interactome", an interacting network of proteins that facilitate cellular function (Bonetta, 2010) and rather than just finding candidate genes

consider how it all works together. This study highlights the importance of the interaction of the environment on the biology of the mosquito. Although the genetic background may be present, the efficacy of the resistance phenotype is modulated by larval nutrition (chapter 3, figure 3.3.2). Multiple blood meals affect the activity of metabolic detoxification enzymes over time (chapter 5, figure 5.3.4 and figure 5.3.5). These are all factors that are not adequately addressed by not considering the interaction between molecular biology and the environment. In practical terms, operational vector control programmes do not have the time or resources to consider the entire interactome whenever a decision needs to be made. The importance of this study is that it highlights how basic research may eventually form a scaffold for future operational activities.

Malaria elimination will not be a once off event because the global approach is a sequential one. Elimination in any region will need to be maintained because of the threat of re-invasion from adjacent areas. Vector-borne diseases present a particular challenge for public health because there are three components in the disease transmission cycle (vector-parasite-human) rather than typical two (disease agent-human). The dynamic nature of the insect pest is also a particular challenge, as there are many aspects of vector biology, including its' molecular makeup as well as its' response to external effects such as climate change. Studying the vector within the context of an interactome (DNA-RNA-protein-physiology-ecology) will ultimately lead to better vector control efforts as it will enable better efforts to target the pest by disrupting elements of the interactome that would benefit the vector. This could be crucial for the maintenance of malaria elimination.

From the data gathered in this study it can be concluded that for the purpose of resistance management, insecticide resistant and susceptible *Anopheles* mosquitoes need to be considered as different biological entities, rather than just organisms that differ by the expression of resistance-related genes. If data from *Culex* mosquitoes were examined in isolation, it would be easy to draw the conclusion that, under situations without an insecticide selection pressure, insecticide resistant individuals constitute the less fit proportion of the population. The data from *Anopheles* populations complicates this simple conclusion. The first of these findings was that pyrethroid resistance did not incur a fitness cost in *An. funestus* (Okoye *et al*., 2007) and the present study has demonstrated that insecticide resistant individuals, particularly the *An. arabiensis* strain SENN DDT is remarkably tolerant of stress.

Elevated stress tolerance is a continuous theme throughout this study. As there are no resistance mechanisms, susceptible *An. arabiensis* become even more sensitive to insecticide intoxication after larval nutritional deprivation (chapter 3, figure 3.3.2). Insecticide resistant *An. arabiensis* and *An. funestus* are more tolerant of oxidative stress (chapter 4, figures 4.3.4-4.3.6), and this has the advantage in *An. arabiensis* in that it enhances the resistant phenotype in the senescing female as a consequence of multiple blood feeding (chapter 5, figure 5.3.1). Coupled with the fact that insecticide resistant *An. arabiensis* are generally bigger (chapter 3, figure 4.3.3 and figure 4.3.4), the only fitness disadvantage found in this study is that resistant individuals typically have a reduced lifespan (chapter 5, figure 5.3.9).

It is therefore in marked contrast to *Culex* mosquitoes that, within the parameters of this study, insecticide resistant *Anopheles* mosquitoes are considerably fitter than their susceptible counterparts. The fact that they appear to be more stress tolerant is of particular concern. That they benefit more from multiple blood meals (chapter 5, figure 5.3.6-5.3.9) is also of particular concern. In the context of maize agriculture in Africa, which benefits *An. arabiensis* larvae, the fact that females that take multiple blood meals sustain their resistance phenotype may have significant epidemiological consequences.

It is for this reason that a paradigm shift is needed when considering insecticide resistant anophelines. In the context of the principle of an interactome, it is necessary to think of what other implications come with the detection of insecticide resistance. This study raises the concern that it not only has implications on the choice of insecticide to be used, but rather that there is an altogether more difficult vector to deal with. This also ties in with with a recent review on the role of behavioural adaptions in malaria control interventions. In this review Gatton *et al.* (2013) highlight the fact that the molecular basis of resistance is easier to study as it is more easily examined under laboratory conditions. This strategy, which underpins the Global Plan for Insecticide resistance management (GPIRM), does not take into account the effectof variable behaviourable adaptation of insecticide resistant individuals in contrast to their susceptible counterparts (Gatton *et al*., 2013). As such, this study also highlights the importance of integrated efforts in vector control research.

In conclusion, environmental stress plays a critical role in the biology of the malaria vector species *An. arabiensis* and *An. funestus*. Insecticide resistance phenotypes, as well as life history, are strongly affected by larval environmental history and oxidative stress. Although

most of these studies require validation by field studies, the body of evidence from the work presented in this study has implications for vector control. The largest of these is that insecticide resistance may have more far reaching consequences than previously thought. This work highlights the dynamic nature of the construction of the insecticide resistance phenotype, as well as how basic research can form a scaffold for future vector control endeavours.

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Appendix 1: Published Manuscipts

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MALARIA JOURNAL

RESEARCH Open Access

The effect of larval nutritional deprivation on the life history and DDT resistance phenotype in laboratory strains of the malaria vector *Anopheles arabiensis*

Shüné V Oliver[1,2] and Basil D Brooke[1,2*]

Abstract

Background: *Anopheles arabiensis* is a major malaria vector in Africa. It thrives in agricultural areas and has been associated with increased malaria incidence in areas under rice and maize cultivation. This effect may be due to increased adult size and abundance as a consequence of optimal larval nutrition. The aim of this study was to examine the effect of larval nutrition on the life history and expression of insecticide resistance in adults of laboratory reared *An. arabiensis*.

Methods: Larvae drawn from an insecticide susceptible *An. arabiensis* strain (SENN) as well as a DDT-resistant strain (SENN-DDT) were subjected to three fasting regimes: 1 mg of food per larva offered once per day, once every second day and once every third day. Control cohorts included larvae offered 1 mg food thrice per day. The rate of larval development was compared between matched cohorts from each strain as well as between fasted larvae and their respective controls. The expression of DDT resistance/tolerance in adults was compared between the starved cohorts and their controls by strain. Factors potentially affecting variation in DDT resistance/tolerance were examined including: adult body size (wing length), knock-down resistance (*kdr*) status and levels of detoxification enzyme activity.

Results and conclusion: *Anopheles arabiensis* larval development is prolonged by nutrient deprivation and adults that eclose from starved larvae are smaller and less tolerant to DDT intoxication. This effect on DDT tolerance in adults is also associated with reduced detoxification enzyme activity. Conversely, well fed larvae develop comparatively quickly into large, more DDT tolerant (SENN) or resistant (SENN-DDT) adults. This is important in those instances where cereal farming is associated with increased *An. arabiensis* transmitted malaria incidence, because large adult females with high teneral reserves and decreased susceptibility to insecticide intoxication may also prove to be more efficient malaria vectors. In general, larval nutrient deprivation in *An. arabiensis* has important implications for subsequent adults in terms of their size and relative insecticide susceptibility, which may in turn impact on their malaria vector capacity in areas where insecticide based control measures are in place.

Keywords: *Anopheles arabiensis*, Larval nutrition, DDT resistance, Adult body size

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Background

The "silver spoon" hypothesis of larval nutrition in holometabolous insects suggests that well nourished larvae become healthier adults that are better capable of survival in variable environments [1]. Larval food restriction in mosquitoes exerts several effects. These include a longer developmental period, with larvae taking a longer time to pupate [2]. A prolonged larval stage is generally associated with an increased risk of mortality as a consequence of predation, breeding site instability and/or human interference [3]. Adults that eclose from larvae with low teneral reserves are smaller [4] and require more blood-meals to produce eggs [5].

The effect of larval starvation on mosquito-borne disease transmission is a subject of debate. It is postulated that larger mosquitoes live longer, increasing their disease transmission potential [6]. It is also reported that a restricted larval diet resulted in a modest but significant increase in the lifespan of the yellow fever vector *Aedes aegypti* [7]. In malaria vectors, smaller mosquitoes are postulated to have greater concentrations of free malaria parasites in their systems because they are less capable of melanising them, resulting in a higher transmission potential [8-10]. Furthermore, the multiple blood-meals required by smaller mosquitoes results in increased host contact and a greater risk of disease transmission [11].

Anopheles arabiensis is one of the dominant malaria vectors in Africa, particularly in Southern and Eastern Africa [12]. It is exophilic and not as anthropophagic as its sibling vector, *An. gambiae*. Although not as efficient a vector as *Anopheles funestus*, with which it is often found in sympatry, it is as efficient a vector as *Anopheles gambiae* S form [13]. As a consequence of exophily, it is a difficult vector to control by indoor residual spraying and the use of insecticide-treated bed nets [14].

Anopheles arabiensis is associated with the "paddies paradox". This occurs in those instances where malaria transmission is reduced in irrigated areas compared to non-irrigated areas, likely because *An. arabiensis* tend to replace the more efficient vector *An. funestus* in irrigated areas [15]. For example, rice farming has been shown to exert a significant effect on malaria vector population dynamics whereby *An. arabiensis* are most abundant when rice crops are immature [16] and after fertilising [17]. Where *An. arabiensis* and *An. funestus* occur in sympatry, planned agriculture appears to lower the incidence of *An. funestus* borne malaria, but tends not to affect *An. arabiensis* borne malaria [18].

The most dramatic example of agriculture on the life history of *An. arabiensis* concerns the effect of maize farming. Maize is a ubiquitous African crop which flowers during rainy seasons. *Anopheles arabiensis* larvae are enhanced by the presence of maize pollen [19], so much so that it even makes up for the effect of larval crowding [20]. Larvae fed on maize pollen alone produce adults comparable in size to laboratory-reared adults fed on optimal diets [19]. It is postulated that increased malaria incidences in maize-growing districts are due to pollen abundance in areas close to where the vectors breed. The larvae readily ingest the pollen which promotes growth. Maize is commonly grown near dwellings allowing healthy adult mosquitoes to participate in malaria transmission [19].

The effect of larval nutrition on the expression of insecticide resistance in anophelines remains largely unexamined. It has previously been shown that adult *Musca domestica vicinia* flies raised on goats' milk are more resistant to DDT than those raised on cow's milk or other diets. *Aedes aegypti* larvae reared on a diet of milk are slightly more susceptible to insecticides than those raised on wheat flour [21]. From studies in mammals such as rats, it has been shown that micronutrients such as niacin, riboflavin, pantothenic acid and pyridoxine are required for Phase I reactions, specifically the cytochrome P450 system. Phase II conjugation reactions are also dependent on various nutritional requirements [22]. It is, therefore conceivable that metabolically-mediated insecticide resistance can be affected by generalized nutritional deprivation induced by starvation. The aim of this study was to examine the effect of generalized nutrient deprivation by starvation on the development of *An. arabiensis* larvae as well as on the expression of insecticide resistance in the resulting adults.

Methods

Mosquito strains

The SENN *An. arabiensis* strain was colonized from material collected in Sennar, Sudan, in 1980 and was used as the baseline for this study. A DDT resistant strain, called SENN-DDT, was intensively selected from SENN. Although selected for resistance to DDT, SENN-DDT also shows resistance to permethrin, deltamethrin and malathion. SENN-DDT is fixed for the L1014S *kdr* mutation [23]. This sodium channel mutation results in reduced neuronal sensitivity to DDT and pyrethroids, leading to DDT and pyrethroid cross resistance [24]. Both strains are maintained under standard laboratory conditions as described in Hunt *et al.* [25] in the Botha DeMeillon insectary facility at the National Institute for Communicable Diseases/NHLS in Johannesburg.

Larval fasting regime

A fasting regime was established for the larvae of both strains. In the first cohort, larvae were provided with the equivalent of 1 mg of food (powdered dog food and yeast [25]) per larva per day (Daily cohort). Second cohort larvae were provided with a single aliquot of food on alternate days (2 Day cohort) and third cohort larvae

were fed once every three days (3 Day cohort). Control larvae were provided with 1 mg of food per larva three times per day (Control cohort).

Larval development rates

The larval fasting regimes described beforehand were performed and the development of the larvae monitored. For each larval fasting regime described above as well the control feeding regime fifty first instar larvae were placed in distilled water. The development rate of the larvae was quantified by recording the time to emergence of the first pupae as well as the time to emergence of 50% or more of the pupae. The larval fasting regimes were implemented for the SENN and SENN-DDT strains. Ten replicates were performed for each of the three fasting regimes for each strain as well as the controls.

For all subsequent experiments larvae were mass reared either under the control feeding regime or the 2 day fasting regime for comparisons between the two. Adults that accrued were used to determine adult susceptibility to DDT, adult size (wing length), *kdr* status and enzyme activity. The 2 day feeding regime was selected because it proved to be the regime most reduced that still allowed an appreciable number of adults to emerge for subsequent analysis. Adults from the 3 day feeding regime had a high level of mortality during eclosion and were therefore not suitable for further analysis.

Adult susceptibility to DDT

Lethal time to induce either 50% or 75% mortality (LT50/LT75) by exposure to DDT was determined by bottle bioassay for each cohort by gender [26]. In brief, 1 mg/ml technical grade DDT (Sigma Aldrich, 31041) was prepared in acetone and used to coat the inside surfaces of 250 ml glass bottles at a concentration of 1 mg DDT per bottle. Three day old non-blood fed adults were exposed to DDT for varying lengths of time: 2.5, 5, 10, 15, 20, 25, 30 minutes for the base SENN strain, and 2, 4, 8 and 16 hours for the resistant SENN-DDT strain. Males and females were exposed separately with 20 adults being used per bottle for each time interval. For the SENN strain 50 replicates were performed per time interval and for the SENN DDT strain 25 replicates were performed per time interval. After each time interval, the adults were removed from the bottle, placed into cups and provided with a 10% sucrose solution. Mortality was scored 24 hours post exposure. Control exposures included the use of bottles treated with acetone only. An environmental control consisted of unexposed mosquitoes in cups. Data were log transformed to allow for the calculation of lethal time (LT) using linear regression. The LT75 was calculated for each cohort of the susceptible SENN strain as LT50 was too short a period to calculate accurately for this strain.

Determination of adult size and *kdr* status of each strain

Three factors were examined in order to explain the difference in DDT resistance levels between control and fasted cohorts: vigour tolerance as approximated by adult size, *kdr* status and metabolic enzyme activity.

Wing length was used as an approximation of adult size [8] and was used to determine whether adults that succumbed to DDT exposure differed significantly in size from those that survived the same treatment. The sizes of SENN adults from the 2 day larval feeding regime cohort that survived 2.5 minutes exposure to DDT (collected 24 hours post exposure) were compared to those that proved susceptible following 2.5 mins DDT exposure (also collected 24 hours post exposure). This procedure was also performed on the SENN control cohort. The sizes of SENN-DDT adults from the 2 day larval feeding regime cohort that survived a 16 hour exposure to DDT (collected 24 hours post exposure) were compared to those that proved susceptible following a 16 hour DDT exposure (also collected 24 hours post exposure). As with the SENN strain, the procedure was also performed on the control cohort. Fifty individuals were measured for each strain treatment and gender. Both wings from each test mosquito were removed and placed on a glass slide. Wing length was determined using a calibrated micrometer at 200X magnification by measuring the distance from the distal end of the alula to the tip, excluding fringe scales [27]. Wing lengths of the exposure survivors were compared to those that had died. Differences in wing lengths were also analysed between samples from the different fasting regimes and gender.

SENN-DDT adults exposed to DDT for the longest time period (16 hours) were preserved, with the survivors and the dead preserved separately for *kdr* analysis. Similarly, the survivors and the dead of the shortest exposure (2.5 minutes) for the susceptible SENN strain were preserved. Thirty individuals were collected from each of these four phenotyped groups. DNA was extracted from each whole specimen using the method described by Collins *et al.* [28]. The presence of the L1014F (*kdr West*) and L1014S (*kdr East*) point mutations was analysed by hydrolysis probe analysis as described by Bass *et al.* [24].

Detoxification enzyme activity in association with larval nutrition

Absolute detoxification enzyme activities for the two-day fasted and control SENN and SENN-DDT adults were determined. For each gender and strain 50 three day old non-bloodfed adult females and males were assayed. Their enzyme activities were compared to the standard susceptible *An. arabiensis* KGB strain. The adults were

homogenised in distilled water and used in subsequent enzyme assays. Proteins were quantified using the Bradford assay, with Bovine Serum albumin as a standard curve [29]. Cytochrome P450 activity was measured as a function of haeme peroxidase activity [30]. The activity of α and β-esterases as well as general glutathione S-transferase (GST) activity was also determined according to established protocols [31,32].

Data analysis

Exposure data were not corrected by Abbott's formula as all control mortalities were below 5%. All statistical analysis was performed using Statistix 7. Comparisons between two samples were analysed using 2 sample t-tests. Comparisons between multiple samples were performed using one way Analysis of Variance (ANOVA) with the Tukey comparison of means being used as a post hoc test. Significance was set at 95% confidence.

Results

Larval development

Dietary restriction was generally associated with a decrease in the rate of larval development (Figure 1). The rate of larval development in the SENN cohorts fed once daily did not differ significantly from that of the control cohorts, but those SENN cohorts fed on alternate days and every third day took significantly longer for the first pupae to appear (ANOVA: $p < 0.01$, $F = 235$, $df = 39$). In SENN-DDT, however, only the cohort fed every third day developed significantly slower than the control (ANOVA: $p < 0.01$, $F = 116$; Tukey; $Q = 3.81$; $df = 39$). Comparing the control cohorts as well as cohorts fed daily between strains, SENN larvae developed significantly faster than SENN DDT larvae (ANOVA: $p < 0.01$, $F = 19.7$, $df = 89$). In the cohorts fed once either every two or three days, there was no significant difference in the rate of development between SENN and SENN-DDT.

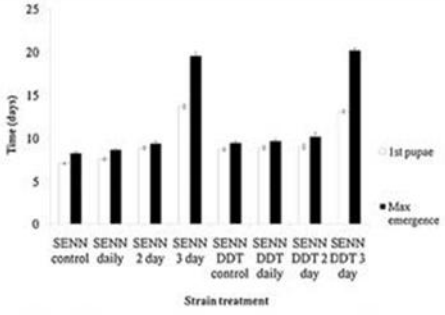


Figure 1 Larval development times (expressed as days to first pupations and days to 50% pupation) by larval feeding cohort for the *Anopheles arabiensis* SENN and SENN-DDT laboratory colonies. Cohort feeding regimes were thrice daily for the controls, once daily (SENN and SENN-DDT daily), once every second day (SENN and SENN-DDT 2nd day) and once every third day (SENN and SENN-DDT 3rd day). A total of 500 larvae per treatment were monitored, divided into 50 larvae per replicate.

Adult susceptibility to DDT

Adults that emerged from fasted larvae tended to have a reduced lethal time (LT) to 50% or 75% mortality during exposure to DDT compared to those from the controls. SENN fasted males showed a significantly reduced LT75 (2-sample t test: $p < 0.01$; $t = 5.524$), as did fasted females ($p < 0.01$; $t = 13.51$) (Figure 2A). This trend was partially mirrored in SENN-DDT (Figure 2B), where only fasted females showed a significantly reduced LT50 ($p = 0.02$, $t = 3.54166$). There was no significant difference in DDT susceptibility between SENN control males and females or between SENN fasted males and females. There was a significant difference in resistance to DDT between control SENN-DDT males and females (2-sample t test: p =

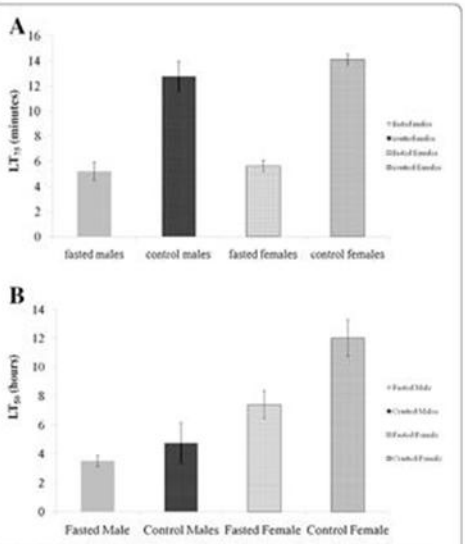


Figure 2 (A) Mean lethal times (minutes) required to induce 75% mortality (LT75) in adult SENN *Anopheles arabiensis* by exposing them to DDT at a concentration of 1mg per 250 ml glass bottle. Adults eclosed from fasted larvae (fed once every second day) are compared to adults eclosed from control larvae (fed thrice per day) by gender. (**B**) Mean lethal times (hours) required to induce 50% mortality (LT50) in adult SENN-DDT *Anopheles arabiensis* by exposing them to DDT at a concentration of 1mg per 250 ml glass bottle. Adults eclosed from fasted larvae (fed once every second day) are compared to adults eclosed from control larvae (fed thrice per day) by gender.

0.02; t = 3.542) as well as between SENN-DDT fasted males and females (2-sample t test: p = 0.02; t = 3.744).

Adult sizes

Control SENN survivors following exposure to DDT were significantly larger than those that succumbed by gender (ANOVA: $p < 0.01$; $F = 40.2$; $df = 199$) (Figure 3A). This was partially true for SENN-DDT, where only the female survivors were significantly larger than those that died (ANOVA: $p < 0.01$; $F = 9.05$, $df = 199$) (Figure 3B). Generally, fasted mosquitoes of either gender were smaller than their corresponding controls (Figures 3A and 3B) (ANOVA females: $p < 0.01$; $F = 28.4$; males: $p < 0.01$; $F = 61.1$; $df = 199$). Relative sizes of fasted adults in relation to their control counterparts are illustrated in Figure 4.

Kdr status of each strain

The L1014S mutation was not detected in either the SENN or SENN-DDT strains. All SENN-DDT samples assayed for the L1014F mutation were homozygous resistant (L1014F/F), regardless of larval nutritional regime or whether they survived DDT exposure or not. All SENN adults were homozygous susceptible (wild-type L1014L/L), regardless of larval nutritional regime.

Detoxification enzyme activity in association with larval nutrition

The mean absolute enzyme activities of each stain by feeding regime are given in Table 1. SENN control cohort enzyme activities of each enzyme class did not differ significantly from those of the corresponding KGB strain by gender, while the SENN-DDT control cohorts showed significantly higher enzyme activity levels for cytochrome P450 (ANOVA: $p < 0.01$, $F = 19.1$ for females; $p < 0.01$, $F = 22.7$ for males; $df = 489$), GST (ANOVA: $p = 0.02$, $F = 4.28$ for females; $p < 0.01$, $F = 15.4$ for males; $df = 489$), α-Esterase (ANOVA: $p < 0.01$, $F = 8.79$ for females; not upregulated in SENN-DDT males; $df = 489$) and β-esterase (ANOVA: $p < 0.01$, $F = 9.89$ for females; $p < 0.01$, $F = 25$ for males; $df = 489$). Female SENN-DDT controls generally showed the highest enzyme activity levels. When protein concentrations were standardised, fasted adults generally showed decreased rates of enzyme activity although not all enzyme classes were significantly decreased

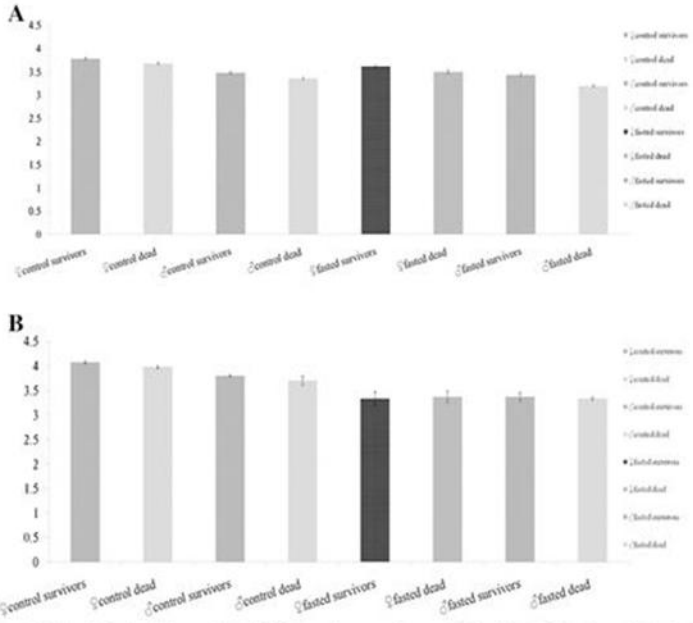


Figure 3 (A) Wing lengths (mm) of *Anopheles arabiensis* SENN survivors and susceptibles (dead) following a 2.5 minute exposure to DDT. Control cohorts (eclosed from larvae fed thrice daily) are compared to their corresponding fasted cohorts (eclosed from larvae fed once every second day) by gender. (**B**) Wing lengths (mm) of *Anopheles arabiensis* SENN-DDT survivors and susceptibles (dead) following a 16 hour exposure to DDT. Control cohorts (eclosed from larvae fed thrice daily) are compared to their corresponding fasted cohorts (eclosed from larvae fed once every second day) by gender.



Figure 4 Example of size variation between SENN *Anopheles arabiensis* adults eclosed from control larvae fed thrice per day (top) and those eclosed from larvae fed every second day (bottom). A = females, **B** = males.

in all cohorts (Table 2). Fasted SENN males showed the greatest decrease in enzyme activity, with three out of four (Cytochrome P450, β esterase and GST) enzyme classes decreased. All other cohorts showed two out of four classes decreased. Cytochrome P450 and β esterase, both Phase I enzymes, were the most down-regulated in the fasted cohorts, with Glutathione S-transferases, Phase II enzymes, the least affected, only being significantly down-regulated in fasted SENN males.

Discussion

In general, food restriction at the larval stages of *An. arabiensis* slows the rate of larval development and affects body size and insecticide tolerance in adults. Similar effects

Table 1 Mean absolute detoxification enzyme activities for the SENN and SENN-DDT *An. arabiensis* strains by larval feeding regime cohort by gender

	Cytochrome P450 equivalents/mg protein	α Esterase - α napthol equivalents/mg protein	β Esterase - β napthol equivalents/mg protein	Glutathione S-transferase µmol/min/ml
	(SD)	(SD)	(SD)	(SD)
KGB♂	0.0046	0.0037	0.0044	0.000072
	(0.0012)	(0.0011)	(0.0008)	(0.00001)
KGB♀	0.0053	0.0048	0.0055	0.000103
	(0.0017)	(0.0013)	(0.0017)	(0.000041)
SBF♂	0.0047	0.0013	0.0049	0.000046
	(0.0018)	(0.0008)	(0.0016)	(0.00002)
SBF♀	0.0047	0.0017	0.0048	0.000048
	(0.0014)	(0.0007)	(0.0013)	(0.000017)
SBC♂	0.006	0.0017	0.0061	0.000057
	(0.0018)	(0.001)	(0.0021)	(0.00003)
SBC♀	0.0063	0.0039	0.0052	0.00007
	(0.0015)	(0.0017)	(0.0015)	(0.000072)
SDF♂	0.0105	0.0012	0.0038	0.000109
	(0.0034)	(0.0006)	(0.0006)	(0.0001)
SDF♀	0.0081	0.0041	0.0063	0.000121
	(0.0027)	(0.0023)	(0.003)	(0.000034)
SDC♂	0.0094	0.0014	0.0077	0.000139
	(0.003)	(0.0007)	(0.0014)	(0.000066)
SDC♀	0.0102	0.0066	0.0068	0.000144
	(0.0035)	(0.0034)	(0.0011)	(0.000041)

The KGB insecticide susceptible *An. arabiensis* strain was used as a baseline comparison against SENN fasted (SBF), SENN Control (SBC), SENN-DDT fasted (SDF) and SENN-DDT control (SDC). SD = standard deviation.

Table 2 Comparison of enzyme activities of SENN and SENN-DDT *An. arabiensis* adults eclosed from fasted larvae (2 day cohort) against their corresponding control cohorts (eclosed from larvae fed thrice daily) by gender

	SENN fasted ♂	SENN fasted ♀	SENN DDT fasted ♂	SENN DDT fasted ♀
Cytochrome P450	↓(0.02)	↓(<0.01)	-	↓(0.02)
Alpha esterase	-	↓(<0.01)	↓(<0.01)	↓(<0.01)
Beta esterase	↓(<0.01)	-	↓(<0.01)	-
Glutathione-S tranferase	↓(0.02)	-	-	-

Statistical significance was assessed by 2 sample *t*-test. Significant decreases (↓) are accompanied by their respective p values in parentheses and hyphens indicate no statistically significant differences.

of larval starvation on development rate and adult size have been recorded in the closely related *An. gambiae* [4].

There was some variation between *An. arabiensis* strains in terms of larval development rate. In particular, SENN control larvae developed faster than control larvae from the resistance selected SENN-DDT strain. This difference may be an inadvertent selection effect caused by intense selection for resistance to DDT in the SENN-DDT strain [33]. Nevertheless, these data show that the primary cause of a prolonged larval development rate in both strains was nutrient deprivation. This effect was most apparent in those larvae fed once every three days, slowing their developmental rate to nearly half that of the controls.

Larval nutrient deprivation significantly reduced subsequent tolerance to DDT in adult females from both strains as well as in SENN males. Surprisingly, the DDT tolerance of SENN-DDT adult males was not adversely affected following larval nutrient deprivation. This may be attributed to a general reduction in DDT tolerance in SENN-DDT males compared to females. The differences in tolerance to DDT between larval feeding regimes in SENN and SENN-DDT and between genders in SENN can be explained, at least in part, in terms of potential DDT resistance mechanisms and variation in adult body size.

SENN adults that died following DDT exposure were significantly smaller than the survivors. Furthermore, fasted adults were generally smaller than their corresponding control cohorts and males were generally smaller than females. Based on these data adult size is affected by gender and larval nutrition and is significantly associated with tolerance to DDT in SENN. These results were only partially mirrored in SENN-DDT where control female DDT survivors were larger than control female susceptibles, control females were generally larger than control males, and the control cohorts were larger than their corresponding fasted cohorts. There was no variation in adult size in association with DDT tolerance and gender in the fasted cohorts of SENN-DDT. This suggests that in the absence of a DDT resistance mechanism the effect of vigour tolerance, here expressed by adult size in which larger mosquitoes are more insecticide tolerant, is more pronounced.

DDT resistance in the selected SENN-DDT strain is likely based on a combination of L1014F *kdr* and enzyme mediated detoxification, although the relative contributions of each cannot be clearly quantified using the evidence currently available. The L1014F mutation has previously been described in *An. arabiensis* from Sudan [34] as well as in the SENN laboratory colony [23] although in both cases there was no clear correlation between L1014F genotype and DDT resistance phenotype. The L1014F mutation is fixed in SENN-DDT, confirming previously described data [35], and all detoxification enzymes classes were significantly up-regulated in SENN-DDT. However, larval nutrient deprivation was associated with significantly lower enzyme activities in adults from both strains, corresponding to reduced tolerance to DDT intoxication. This suggests that enzyme detoxification plays an important role in production of the DDT resistance phenotype in the SENN-DDT colony. Nevertheless, a link between DDT resistance and L1014F *kdr* in the SENN-DDT colony has been established based on the use of synergists [35] and is inferred by selection to fixation of L1014F using DDT.

The link between detoxification enzyme activity and the expression of DDT resistance/tolerance may be explained by examining the physiological effect of larval nutrient deprivation. When larval teneral reserves are depleted the larval fat body rearranges itself. There is a loss of mitochondria and rough endoplasmic reticulum, and there is a global reduction of protein as well as various endocrine disruptions [36]. As these effects are passed through to the adult stage, it is not surprising that adult enzyme activity levels in the starved cohorts are generally decreased, even after correction for total protein content. From the data presented here it is apparent that Phase I enzymes are the most affected, with cytochrome P450s and beta esterases showing the most significant reductions in activity. Interestingly, GST activity appears to be the least affected even though GSTs are primarily associated with resistance to DDT [37,38]. It is possible that the reduced Phase I detoxification enzyme activity in the fasted cohorts resulted in less detoxification by-product available for Phase II biotransformation, as opposed to the change in resistance in association with fasting being due to a direct reduction in Phase II GST-mediated detoxification.

This study was performed on laboratory strains, and a comparison of resistance profiles of specimens found in field conditions with varying access to larval food resources is needed to corroborate the results. Nevertheless, it is concluded that *An. arabiensis* larval development is prolonged by nutrient deprivation and that adults that eclose from starved larvae are smaller and less tolerant to DDT intoxication. This effect on DDT tolerance in adults is also associated with reduced detoxification enzyme activity most likely caused by larval nutrient deprivation. Conversely, well fed larvae develop comparatively quickly into large, more DDT tolerant adults. This could be important in those instances where cereal farming is associated with increased success of *An. arabiensis* as well as increased malaria incidence [19], because large adult females with high teneral reserves and decreased susceptibility to insecticide intoxication may also prove to be more efficient malaria vectors. In general, larval nutrient deprivation in *An. arabiensis* has important implications for subsequent adults in terms of their size and relative insecticide susceptibility, which may in turn impact on their malaria vector capacity in areas where insecticide based control measures are in place.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SVO assisted with the experimental design, conducted the experiments, analysed the data and produced the initial drafts of the manuscript. BDB assisted with the experimental design, analysis and interpretation of data and produced the final version of the manuscript. All authors read and approved the final manuscript.

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The effect of multiple blood-feeding on the longevity and insecticide resistant phenotype in the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae)

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Abstract

Background: *Anopheles arabiensis* is a major malaria vector in Africa. Adult females are likely to imbibe multiple blood meals during their lifetime. This results in regular exposure to potential toxins and blood-meal induced oxidative stress. Defence responses to these stressors may affect other factors of epidemiological significance, such as insecticide resistance and longevity. The aims of this study were to examine the effect of multiple blood-feeding on insecticide tolerance/resistance with increasing age, to assess the underlying biochemical mechanisms for the responses recorded, and to assess the effect of multiple blood-feeding on the life histories of adult females drawn from insecticide resistant and susceptible laboratory reared *An. arabiensis*.

Methods: Laboratory reared *An. arabiensis* females from an insecticide resistant and an insecticide susceptible colony were offered either a single blood meal or multiple blood meals at 3-day intervals. Their tolerance or resistance to insecticide was then monitored by WHO bioassay four hours post blood-feeding. The biochemical basis of the phenotypic response was assessed by examining the effect of blood on detoxification enzyme activity and the effect of blood-meals on detoxification enzyme activity in ageing mosquitoes.

Results: Control cohorts that were not offered any blood meals showed steadily decreasing levels of insecticide tolerance/resistance with age, whereas a single blood meal significantly increased tolerance/resistance primarily at the age of three days. The expression of resistance/tolerance in those cohorts fed multiple blood meals generally showed the least variation with age. These results were consistent following exposure to DDT and pyrethroids but not to malathion. Multiple blood-meals also maintained the DDT and permethrin resistant phenotype, even after treatment females had stopped taking blood-meals. Biochemical analysis suggests that this phenotypic effect in resistant females may be mediated by the maintenance of increased glutathione s-transferase activity as a consequence of multiple blood-feeding. Multiple blood-feeding increased the longevity of insecticide resistant females regardless of their mating status, but only increased the longevity of unmated susceptible females.

Conclusion: These data suggest that multiple blood-feeding confers a competitive advantage to insecticide resistant females by increased longevity and maintenance of the expression of resistance with age.

Keywords: Blood meal, Insecticide resistance, Oxidative stress

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Background

Anopheles arabiensis is a major malaria vector in Southern and Eastern Africa. It is a member of the *An. gambiae* species complex and often occurs in sympatry with the major malaria vectors *An. gambiae sensu stricto, An. coluzzii* and *An. funestus* [1]. Although it is not as efficient a vector as *An. funestus* and *An. gambiae* [1], *An. arabiensis* does present a unique challenge to vector control efforts because it is endophilic and exophilic and contributes significantly to outdoor malaria transmission where it occurs [2]. Exophilic *An. arabiensis* are not susceptible to control by indoor residual spraying and are not well controlled by conventional and long-lasting insecticide treated nets either [3].

Haematophagy in anautogenous mosquitoes, including *An. arabiensis*, has clear reproductive functions. Blood-feeding, however, has various biological consequences not related to egg production. Blood-feeding sets off a variety of biological processes, with many transcriptional changes peaking at 3 hours after feeding [4]. These changes have an impact on characteristics that affect vector control. Previous studies on various mosquito species have demonstrated that insecticide toxicity is reduced after a single blood-meal [5-9]. A disease vector mosquito female requires two meals to be of epidemiological significance and it is highly likely that during her lifetime a female will take multiple blood meals [10]. Therefore, a question arises as to what effects multiple blood-feeding has on ageing mosquitoes.

In terms of malaria epidemiology and vector control there are two important considerations. The first is the effect of blood-feeding on longevity. Small changes in longevity have profound consequences on disease transmission [11]. A combination of blood and sugar as a dietary combination has been shown to provide the best survival in *An. gambiae* [12]. It is important, however, to know whether this holds equally true for insecticide resistant and susceptible populations.

It is also important to determine the effect of multiple blood-feeding on the expression of insecticide resistance in senescent mosquitoes. It is well established that sensitivity to insecticides increases with age [10-16]. This is assumed to be a function of decreasing enzyme activity due to the reduction of soluble proteins with age, particularly in insecticide resistant mosquitoes [14-19]. It has been suggested that the effect of age is more important in the expression of insecticide resistance than larval nutrition [20], and that a recorded decline in DDT resistance with age in *An. gambiae* from Zanzibar was not an effect of undernourishment, but also occurred in natural populations taking regular blood-meals [16]. Furthermore, several studies have demonstrated that blood-feeding does not influence the decline of insecticide resistance in older mosquitoes [21-23]. This has led to the suggestion that ageing may partially restore the efficacy of insecticide based vector control [24].

The aims of this study were to examine the effect of multiple blood-feeding on insecticide tolerance/resistance with increasing age, to assess the underlying biochemical mechanisms for the responses recorded, and to assess the effect of multiple blood-feeding on the life histories of adult females drawn from insecticide resistant and susceptible laboratory reared *An. arabiensis*.

Methods

This study examined the effect of multiple blood-meals on the resistant phenotype up to the age of 21 days. By this age the female will have passed her extrinsic incubation period [25] and will be of epidemiological significance as a potential malaria vector. The underlying biochemical mechanisms for these responses were also examined.

Laboratory strains

Two laboratory strains of *An. arabiensis* were used in these experiments. SENN is an insecticide susceptible strain from Sennar, Sudan. SENN-DDT is a strain selected for DDT resistance from the SENN strain. The SENN-DDT strain was selected by 1 hour exposures of 1 – 3 day old adult mosquitoes to 4% DDT, and survivors were allowed to breed. This procedure was followed through 100 generations prior to these experiments. This selected strain is resistant to DDT, permethrin, deltamethrin and malathion (unpublished data). Resistance is mediated by elevated cytochrome P450, glutathione S-transferase (GST) and general esterase activity. The mosquitoes are also fixed the L1014F *kdr* mutation [26]. All mosquitoes were reared as described by Hunt *et al.* [23].

Experimental set-up

Adult mosquitoes were subjected to one of three dietary regimens. The control groups were not offered blood throughout their lives, and were maintained only on 10% sucrose solution. This constituted the sugar fed control treatment. The second group was maintained on sucrose solution, but were allowed a single blood-meal at the age of 3, 7, 11, 15, 18 or 21 days. This constituted the single blood-meal treatment group. The third group was maintained by feeding on sucrose solution, but allowed blood at the age of 3, 7, 11, 15, 18 and 21 days. At each time point, a sub-section of the population was removed for bioassays. Therefore, the population removed at 7 days of age had 2 blood-meals; the population removed at 11 days had 3 blood-meals etc. This group constituted the multiple blood-fed treatment group. In this study a cohort is defined as a sample drawn from a single egg batch. For each treatment, 3 cohorts were used, and for every age group, three replicates (individual exposures)

were performed. Mosquitoes that were bioassayed were not returned to their respective populations.

Samples were drawn from each dietary treatment at the ages of 3, 7, 11, 15, 18 and 21 days post emergence. Those that were allowed blood in their diet were fed to repletion. Four hours after feeding, individuals were either exposed to insecticide using the WHO bioassay or kept for biochemical analysis. Those kept for biochemical analysis were aged for a further 72 hours to allow those that had taken one or more blood-meals time to digest the blood completely.

Insecticide susceptibility assays subsequent to blood-feeding

For blood-feeding treatments, females were allowed to feed to repletion on the arm of a human volunteer. All procedures performed were approved by the human ethics committee of the University of the Witwatersrand (Clearance certificate M130534). A single, consenting human volunteer was used as a previous study demonstrated that the blood of a human augmented pyrethroid resistance better than that of an anaesthetised guinea pig. Furthermore, the use of a single human volunteer has not been shown to have any effects uniquely related to the volunteer [27]. Throughout their lives, males and females were kept together and allowed to mate. Females were allowed to oviposit twice a week for the duration of the experiment.

Insecticide susceptibility assays (bioassays) were performed on samples drawn from each treatment cohort within the insecticide resistant SENN-DDT strain at each age interval. In the blood-fed cohorts, females were allowed to feed to repletion. Four hours after the meal, samples from all three dietary treatments were exposed to test papers treated with either 4% DDT, 0.75% permethrin, 0.05% deltamethrin or 5% malathion for 1 hour, and mortality was scored 24 hours post exposure according to the standard WHO procedure [28]. Sample sizes are summarised in Table 1. All insecticide treated papers were obtained from the School of Biological Sciences, Universiti Sains Malaysia (USM), Penang, Malaysia, After exposure, females were allowed *ad libitum* access to 10% sucrose. Bioassay controls included samples exposed to untreated papers. Final mortalities 24 h post exposure was compared between treatments by age.

Delayed blood-feeding bioassays

The aim of this experiment was to determine whether multiple blood-meals had a lasting effect on the insecticide resistant phenotype even after the meals were halted. For this procedure, the three dietary treatment regimes of the previous experiment were followed. However, at day 15, the multiple blood-meal treatment was split into two groups. One group was not offered any more blood, and the second group was given another blood-meal at the age of 18 days. At 18 days, the sugar fed group, the single blood meal group, the multiple blood-meal group that had meals up to the age of 18 days and the multiple blood-meal group that had meals up to the age of 15 days (the delayed exposure group) were exposed by WHO bioassays as described above. Final mortalities 24 h post exposure was compared between treatments as well as between the two multiple blood-meal groups.

Biochemical analysis

Insecticide resistance by increased metabolic detoxification is mediated by one or a combination of three enzyme superfamilies; the cytochrome P450s, Glutathione S-transferases (GSTs) and general esterases. Analysis of these enzyme activities give an indication of the mechanism underlying the insecticide resistance [29,30] Recently, ABC transporters have been implicated in insecticide resistance [31,32] but have not been implicated in insecticide resistance in the laboratory strains of *An. arabiensis* used in this study. Previous analysis of the mechanisms underlying the insecticide resistance phenotype in the SENN-DDT strain has been attributed to the *kdr* mutation as well as elevated activities of α- and β-esterases, ctyochrome P450s and GSTs [26]. Therefore, the effect of blood-feeding was examined on these particular enzyme systems.

Biochemical analysis is normally performed on mosquitoes that have not had a blood-meal, as per WHO guidelines [28]. This is because host components of the blood-meal could interfere with the analysis, as the ingested proteins interact with the assay substrates [28]. This study, however, aimed to determine the effect of blood-feeding treatment on the activity of detoxification enzymes with time. To avoid host protein substrate interference, when samples from the different nutritional regimens were assayed for detoxification enzyme activity, specimens were killed 72 hours after their last feeding treatment. This had the two-fold effect of allowing complete digestion of the blood-meal, and also enabled analysis of the lasting effect of the dietary treatment in each individual. Therefore, in this experiment, although the same time points were used as for the bioassays, chronologically the mosquitoes were 3 days older. For example, in the single point detoxification analysis described below the mosquitoes examined are labelled as 15 days old, but when assayed they were 18 days old. This is because the labelling system reflects their meal regimen rather than their age. For detoxification assays, all specimens were homogenised in PCR grade water after being killed at −70°C.

Table 1 Sample sizes of *Anopheles arabiensis* (SENN-DDT) insecticide treatments by age

Treatment	Age	DDT	Permethrin	Deltamethrin	Malathion
Sugar fed	3 days	714	696	695	813
	7 days	476	464	463	543
	11 days	476	464	463	542
	15 days	476	464	463	-
	18 days	476	464	463	-
	21 days	476	464	463	-
Single blood meal	3 days	714	696	695	813
	7 days	476	464	463	543
	11 days	476	464	463	542
	15 days	476	464	463	-
	18 days	476	464	463	-
	21 days	476	464	463	-
Multiple blood meals	7 days	476	465	463	543
	11 days	476	464	463	543
	15 days	476	464	463	-
	18 days	476	464	463	-
	21 days	476	465	463	-
Total:		**8568**	**8354**	**8335**	**4882**

Single point biochemical assays

As DDT and pyrethroid resistance were most strongly affected by multiple blood-meals, the cytochrome P450 and GSTs were analysed for females drawn from the three treatment regimens that were allowed their last meal or only blood-meal at the age of 15 days. Cytochrome P450 activity was determined as a function of haeme peroxidase activity [29]. GST activity was determined as 1-Chloro-2,4-dinotrobenzene (CDNB) conjugation activity [33]. Proteins were quantified using the Bradford method [30]. The GST and cytochrome P450 activities of samples from SENN and SENN-DDT were determined. Enzyme activities between strains by treatment and between treatments within each strain were compared. For all detoxification enzyme analysis 96 individuals were used for age and blood feeding regimen for each strain.

Biochemical analysis of ageing adult mosquitoes

Previous studies have demonstrated that a single blood-meal enhances the expression of pyrethroid resistance in pyrethroid resistant females [9]. As the previous experiment also demonstrated that blood-feeding affected the expression of resistance in the insecticide resistant SENN-DDT strain, it was decided to analyse the effect of blood-feeding on enzyme activity with age in SENN-DDT. As previously described, samples of 50 females drawn from each dietary treatment at each successive age were killed 72 hours after treatment. Males from each age cohort were also killed, and their enzyme activities used for comparison. Proteins were quantified and Cytochrome P450 and GST activities were determined as previously described. Additionally, general esterase activity was also determined [30]. Enzyme activities by age were compared between treatments and genders. For all detoxification enzyme analysis 96 individuals were used for age and blood feeding regimen for each strain.

Longevity

To determine the effect of multiple blood-feeding on the longevity of SENN and SENN-DDT females, newly emerged males and females were separated. Samples of unmated females provided with 10% sucrose solution only served as a control. 30 unmated females were used as the unmated blood-fed cohort. 30 mated females (placed in a cage with 45 males and allowed to mate for the duration of the experiment) served as the mated blood-fed cohort. The mated and unmated cohorts were blood-fed to repletion twice weekly and a moistened filter paper was provided twice weekly to allow for oviposition. To determine whether any effects observed were due to blood-feeding or due to additional protein supplementation, 30 unmated females were provided with 10% sucrose supplemented with 50 mM Bovine Serum Albumen (BSA). These females were not offered any blood-meals. Longevity of each treatment cohort was monitored until all females had died. For each treatment, three technical repeats were performed with three different cohorts, resulting in a total of nine replicates per

treatment. Mean longevity was compared between treatment cohorts.

Statistical analysis

All statistical analyses were performed using Statistix 8 (Tallahassee, Fl, USA) and Statistica 10 (Statsoft, USA). All means were compared using either a 2-sample t-test or a 1-way Analysis of Variance (ANOVA) with a Tukey comparison-of-means as a post-hoc test. All confidence intervals were set at 95%. Abbott's formula was not applied to bioassay data as control mortalities did not exceed 10% at any point. Longevity was assessed using the Kaplan-Meier estimator, using the log-rank test to determine the significance as none of the data were censored.

Results

Bioassays

For all insecticides tested, a single blood meal at the age of three days significantly reduced insecticide-induced mortality relative to sugar-fed females (Figure 1A, B, C and D). Malathion bioassays were not performed after mosquitoes were 11 days old. Mortality for all treatments exceeded 90% at that age, and, although a single blood meal at 3 days and multiple blood meals by 7 days significantly reduced mortality, blood feeding appeared to cease having an effect on susceptibility by then (Figure 1D). For DDT, permethrin and deltamethrin treatments, multiple blood-meals always resulted in significantly lower levels of mortality compared to sugar fed control females of the same age (Figure 1A, B, C). For mosquitoes exposed to DDT, the mortalities of cohorts fed a single meal at the age of 3 days and those fed multiple blood meals did not differ significantly between the ages of 3 to 18 days (1-Way ANOVA: $p = 0.36$; $F = 1.11$ $df = 1$) (Figure 1A). For permethrin-exposed females, there was no significant variation in mortality in the multiple-fed cohort between the ages of 7–21 days (1-Way ANOVA: $p = 0.35$; $F = 1.13$, $df = 1$) (Figure 1B).

Single blood meals induced a variable effect on mortality, only consistently showing significantly reduced mortality compared to controls in 3 day old mosquitoes (Figure 1A, B, and C, D). Notable exceptions to this single blood meal trend are the significant increase in permethrin-induced mortality at 18 and 21 days (1-Way ANOVA: $p < 0.05$ $F = 6.35$; $df = 1$) (Figure 1B), and the significantly reduced deltamethrin mortality at the age of 18 and 21 days (1-Way ANOVA: $p < 0.05$; $F = 4.98$; $df = 1$) (Figure 1C).

As mosquitoes age, they are more likely to die when exposed to insecticide. In Figure 2, a strong linear relationship between mortality and age is apparent, particularly in sugar-fed individuals exposed to DDT (Pearson's correlation $r = 0.9$; $p < 0.05$); permethrin (Pearson's correlation $r = 0.9$; $p < 0.05$) and deltamethrin (Pearson's correlation $r = 0.9$; $p < 0.05$). When females were provided with a single blood-meal, there was still a strong linear relationship between age and mortality for permethrin treated females (Pearson's correlation $r = 0.9$; $p < 0.05$) (Figure 2B) and deltamethrin (Pearson's correlation $r = 0.9$; $p < 0.05$) (Figure 2C), but a weaker relationship for those exposed to DDT (Pearson's correlation $r = 0.6$; $p = 0.05$) (Figure 2A). For individuals fed multiple meals, there was a comparatively weak linear relationship between age and mortality for the DDT exposed

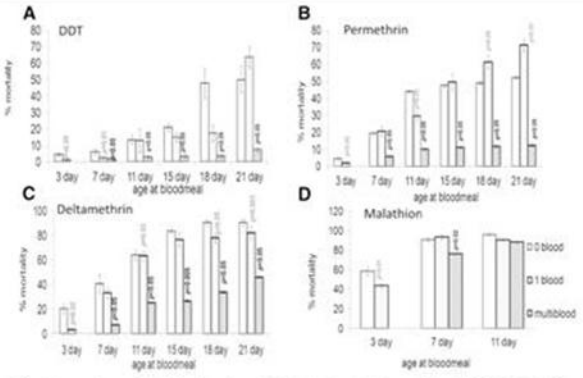


Figure 1 The effect of blood feeding on insecticide induced mortality in *Anopheles arabiensis* SENN-DDT females. Mean percentage mortalities 24 hours post exposure are given for DDT **(A)**, permethrin **(B)**, deltamethrin **(C)** and malathion **(D)**. Insecticide bioassays were conducted 4 hours post blood meal and mortalities are plotted by age and blood feeding regimen i.e. sugar fed only (0 blood), single blood meal (1 blood), multiple blood meals (multiblood). P-values obtained by 2-sample test indicate degree of significance in differences in mortalities between blood-fed cohorts and their respective sugar fed control cohorts.

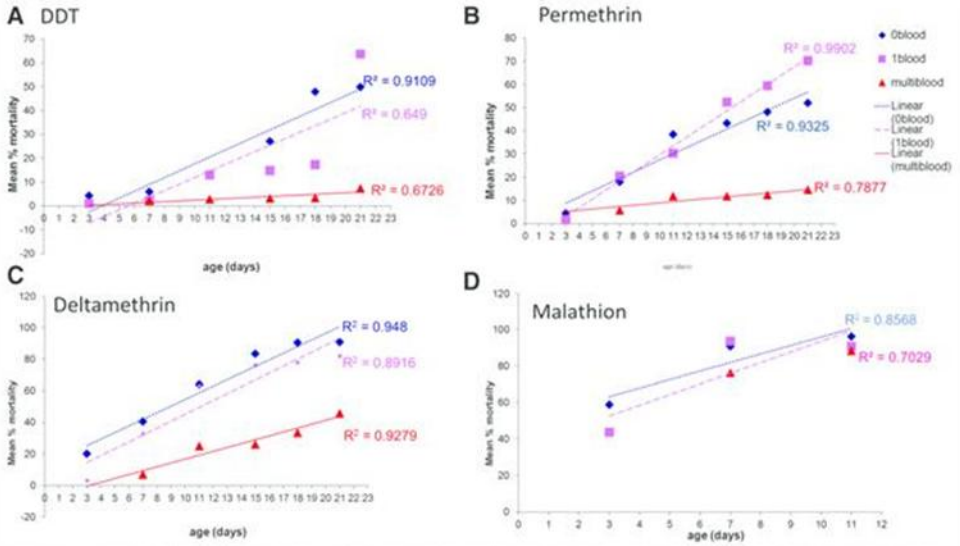


Figure 2 Linear regression analysis of insecticide-induced mortality by age by blood feeding regimen (sugar fed only - 0 blood, single blood meal - 1 blood, multiple blood meals – multiblood) in *Anopheles arabiensis* SENN-DDT females. Insecticide bioassays were conducted 4 hours post blood meal at each age interval indicated and mean percentage mortalities 24 hours post exposure are shown for DDT **(A)**, permethrin **(B)**, deltamethrin **(C)** and malathion **(D)**. Goodness-of-fit for each regression is indicated by R^2 values. No R^2 value is given for multiple-feeding for the malathion treatment as only two points were available for analysis.

cohorts (Pearson's correlation r = 0.6; p = 0.08), whilst for deltamethrin treated cohorts, mortality increased in a linear fashion with age (Pearson's correlation r = 0.9; p < 0.05). In Table 2 it is apparent that for DDT, permethrin and deltamethrin treated individuals mortality of the single blood-meal treated individuals did not vary with age compared to those cohorts that were not offered blood, but the mortalities of those that had multiple blood-meals were significantly lower. Statistical indicators are summarised in Table 2.

Following experiments to determine whether multiple blood meals had an effect on mortality in the long term or only after each meal, it was shown that mortality in the delayed exposure group did not differ significantly from those fed multiple meals in the cohorts exposed to either DDT (2 sample t-test: p = 0.10; t = −1.80) or permethrin (2 sample t-test: p = 0.88; t = −0.15), but was significantly higher in cohorts exposed to deltamethrin (2 sample t-test: p < 0.05; t = −8.94) (Figure 3).

Detoxification enzyme analysis

The cytochrome P450 and GST activity of 15 day old females allowed 72 hours to digest their blood meal (age at analysis is therefore 18 days) were analysed to determine how the effects of multiple blood feeding on the resistance phenotypes are mediated. Cytochrome P450s and GSTs were analysed in particular as the multiple blood meals had a sustained effect on pyrethroid and DDT resistance. Haeme peroxidase activity, a measure of Cytochrome P450 activity, was significantly reduced in SENN-DDT females that had multiple blood meals (2-sample t test: p = 0.01; t = 2.75) (Figure 4A). GST activity in SENN-DDT females that had multiple blood meals was significantly higher than females of the same

Table 2 One way ANOVA comparing insecticide-induced mortality of unfed *Anopheles arabiensis* SENN DDT females at different ages to their counterparts fed either a single or multiple blood meals

	DDT	Permethrin	Deltamethrin	Malathion
1 blood	p = 0.64 (F = 0.23;df = 1)	p = 0.83 (F = 0.05;df = 1)	p = 0.61 (F = 0.26;df = 1)	p = 0.78 (F = 0.09;df = 1)
Multiblood	p = 0.04 (F = 5.16;df = 1)	p = 0.02 (F = 8.56;df = 1)	p = 0.039 (F = 6.76;df = 1)	p = 0.98 (F = 0.00;df = 1)

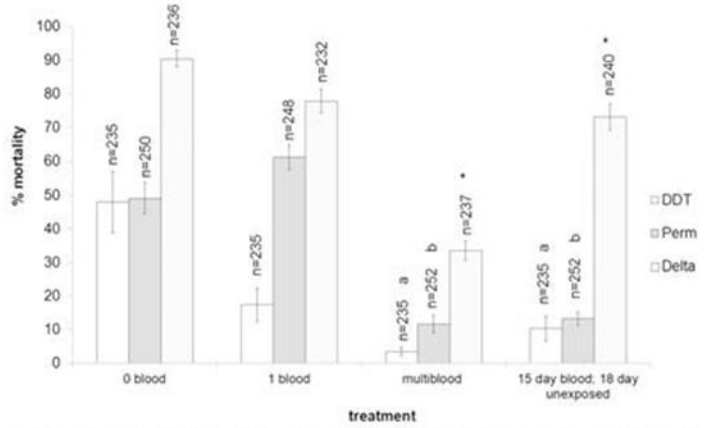


Figure 3 The lasting effects of multiple blood-feeding on the expression of insecticide resistance in *Anopheles arabiensis* SENN-DDT females. Mean percentage mortalities of 18 day old SENN-DDT females that either had no blood, a single blood meal (1 blood) or their fifth blood meal (multiblood) 4 hours prior to exposure were compared to that of 18 day old females that acquired their last blood meal at the age of 15 days (15 day blood; 18 day exposed). Insecticide bioassays were conducted 4 hours post blood meal except for the last cohort which was assayed against listed insecticides 3 days post blood-feeding (15 day blood; 18 day exposed). Only the mean mortality induced by the delayed deltamethrin treatment differs significantly from the mean mortality of the multiple blood meal cohort (multiblood) of the same age ($p < 0.05$). The effect of multiple blood-feeding on the expression of resistance was maintained for the DDT and permethrin resistant phenotypes 3 days post blood-feeding. Perm = permethrin; Delta = deltamethrin. The asterisks denote significant differences between 18 day multiple blood meal treatments and those exposed at 18 days but last fed 3 days prior to exposure. Lower case letters indicate no significant differences.

strain and age that had not been offered blood (2-sample t-test: $p < 0.05$, $t = -7.74$), as well as females that had taken a single blood meal (2-sample t-test: $p < 0.05$, $t = -11.88$). GST activity in SENN-DDT females that had multiple blood meals was significantly higher than SENN females that had no blood (2-sample t-test: $p < 0.05$, $t = 9.22$), a single blood meal (2-sample t-test: $p < 0.05$, $t = 9.44$) or multiple blood meals (2-sample t-test: $p < 0.05$, $t = 6.86$). When compared to unfed 3-day old SENN-DDT females, 18-day old SENN-DDT females that had taken multiple meals showed significantly higher levels of GST activity (2 sample t-test $p = 0.01$; $t = -2.59$) (Figure 4B). In contrast, although multiple blood meals significantly increased GST activity in SENN females compared to those of the same age that had no blood meals (2 sample t-test: $p < 0.05$; $t = -17.31$) or a single meal (2 sample t-test: $p < 0.05$; $t = -12.54$), the GST activity of unfed 3 day old females had significantly higher levels of GST activity than those that had multiple blood meals (2 sample t-test: $p < 0.05$; $t = -5.99$).

Multiple blood meals exerted a significant effect on the detoxification enzyme activity of insecticide resistant, but not susceptible females. An analysis of detoxification enzyme activity over time as affected by blood meals was therefore conducted on SENN-DDT females only, with males of the same age used as a comparison (Figure 5). The same age intervals were used as for the bioassays, with 72 hours allowed for blood digestion. Therefore, although the ages of 3 until 21 days are indicated, analysis actually took place 3 days afterwards. The Cytochrome P450 activity of non blood-fed females was always significantly higher than those that had either a single or multiple meals (1-way ANOVA $p < 0.05$; $F = 37.9$; $df = 2$) (Figure 5A). Females that had blood meals, either single or multiple, always had significantly lower levels of Cytochrome P450 activity than their male counterparts (1-way ANOVA $p < 0.05$; $F = 13.9$; $df = 3$). Only at the age of 18 days (1-way ANOVA $p < 0.05$; $F = 27$; $df = 1$) do the single and multiple blood meal cohorts differ significantly from each other. The GST activity (Figure 5B) of females that had multiple blood meals declined steadily throughout their lives, but always remained significantly higher than those of males or females that had one or no blood meals (1-way ANOVA $p < 0.05$; $F = 37.9$; $df = 3$). A single blood meal at the age of 3 days significantly increased both α- and β-esterase activity (2 sample t-test: $p < 0.05$; $t = 5.63$ for α-esterases and $p < 0.05$; $t = -4.48$ for β-esterases). Multiple blood meals significantly increased α- and β-esterase activity from the age of 15 days compared to the no blood, single blood and male cohort (1-way ANOVA: $p < 0.05$; $F = 20.4$; $df = 3$ for α-esterases and $p < 0.05$; $F = 15.8$; $df = 3$ for β-esterases) (Figure 5C; Figure 5D).

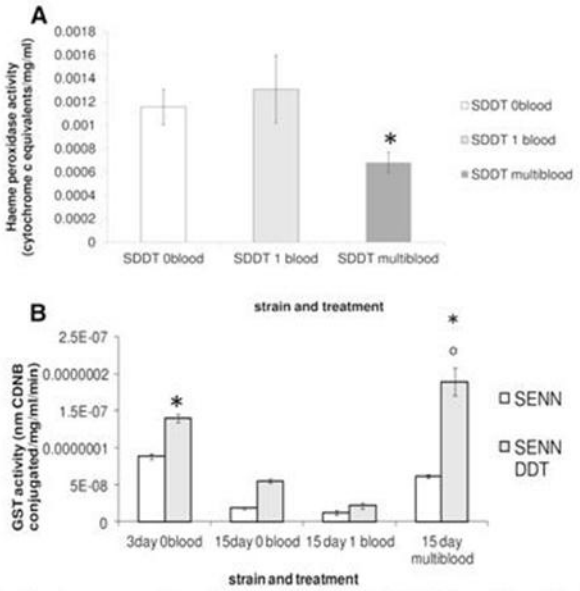


Figure 4 Comparison of detoxification enzyme activity of 18 day old SENN and SENN-DDT *Anopheles arabiensis* females. 15 day old females either deprived of blood (15 day 0 blood), offered only one blood meal at 15 days (15 day 1 blood) or fed multiple blood meals at three day intervals up to and including 15 days (15 day multiblood) were allowed 72 hours to digest their final blood meal (if offered) before being assayed for detoxification enzyme activity. **Block A** represents haeme peroxidase activity and shows a comparison of SENN-DDT females that had no blood meals compared to those that had single or multiple meals. The asterisk denotes a significantly lower enzyme activity in multiple blood-fed SENN-DDT compared to all other cohorts. SENN is not represented as there were no significant changes in haeme peroxidise activity in this strain regardless of dietary treatment. **Block B** represents Glutathione S-transferase (GST) activity of SENN and SENN-DDT females. The asterisks denote a significant difference between GST activities of the different strains at the same age. A circle denotes a significant increase in activity of a 15 day old SENN-DDT cohort compared to their 3 day old counterparts.

Longevity

Blood-feeding had a differing response on resistant and susceptible strains. BSA was used as a control to determine whether the effects induced by blood meals were due to blood itself, or simply additional dietary protein. BSA significantly reduced the longevity of SENN females compared to those that only had a sugar meal (Log rank test: $p = 0.04$; $\chi^2 = 8.68$) (Figure 6A), but not SENN-DDT females (Log rank test: $p = 0.92$; $\chi^2 = 0.01$) (Figure 6B). Multiple blood meals significantly increased the longevity of mated (Log rank test: $p < 0.05$; $\chi^2 = 13.74$) and unmated SENN-DDT females (Log rank test: $p < 0.05$; $\chi^2 = 11.21$) (Figure 6B). For SENN females, multiple blood meals increased longevity in unmated females (Log rank test: $p < 0.05$, $\chi^2 = 5.29$), but not mated females (Log rank test: $p = 0.14$, $\chi^2 = 2.22$), (Figure 6A).

Discussion

Blood feeding stimulates a variety of metabolic processes in female mosquitoes, and several blood meals may be taken during a gonotrophic cycle, which may result in the triggered changes occurring in a cyclical pattern [34]. This response is important for the gonotrophic cycle and is likely to affect xenobiotic detoxification as well. This is because blood feeding induces changes in the regulation of detoxification enzymes so as to enable the female to cope with the metabolic challenge posed by blood ingestion [34]. Previous studies have assessed the effect of a single blood meal on the expression of insecticide resistance [8,9], but these data are the first to demonstrate the maintenance of the expression of insecticide resistance as a consequence of multiple blood-feeding.

The *An. arabiensis* insecticide resistant strain used in this experiment, SENN-DDT, mediates resistance by a combination of *kdr* and metabolic mechanisms [26]. In the absence of blood feeding, the expressions of DDT, pyrethroid and malathion resistance decline with age in SENN-DDT despite the presence of *kdr*. However, multiple blood-feeding either arrests or reduces this decline

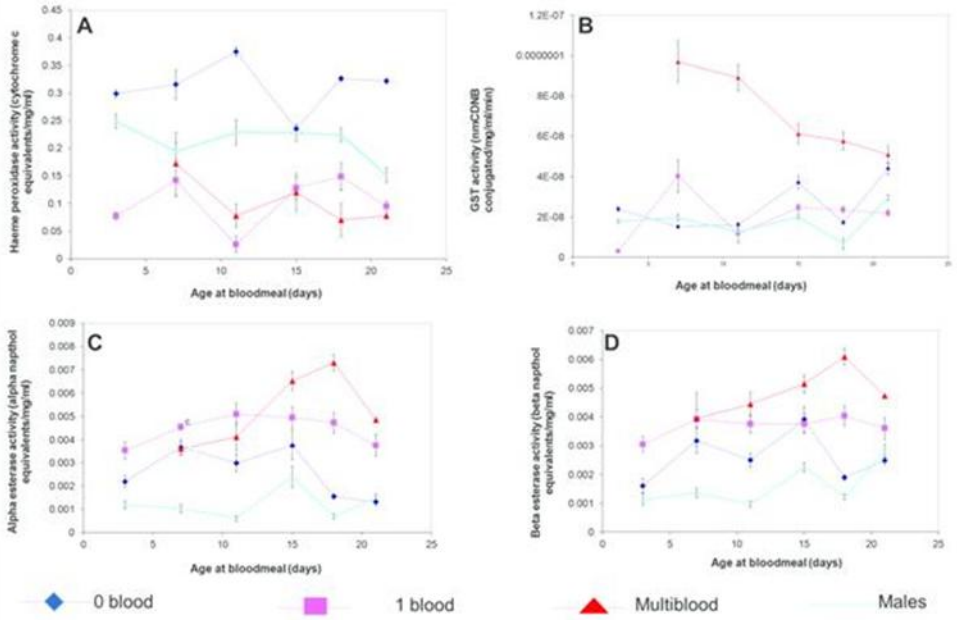


Figure 5 Detoxification enzyme activities of *Anopheles arabiensis* SENN-DDT by age and as a function of blood feeding regimen in females. Block A represents Cytochrome P450 activity as measured by haeme peroxidase activity. **Block B** represents GST activity. **Block C** represents α esterase activity and **Block D** β esterase activity. Enzyme activities in males were measured as baseline levels against which the enzyme activities of unfed, single blood meal and multiple blood meal cohorts of females were compared.

with age. Generally, *kdr* is most closely associated with DDT resistance, followed by Type I pyrethroids, followed by Type II pyrethroids [35,36]. In the SENN-DDT strain, deltamethrin resistance is almost completely (98%) negated by the cytochrome P450 synergist piperonyl butoxide (PBO), suggesting that deltamethrin resistance in this strain is almost exclusively metabolically mediated. This is in contrast to permethrin resistance which is largely mediated by *kdr*, as PBO has a very limited synergistic effect on this phenotype (data not shown). The expression of deltamethrin resistance in SENN-DDT was less affected by multiple blood-feeding than that of DDT and permethrin resistance, while malathion resistance was largely unaffected. This is a confirmation of a previous study noting that blood does not have a strong effect on malathion toxicity [37]. These data suggest that multiple blood-feeding exerts a significant enough effect on the regulation of those detoxification enzymes also associated with insecticide resistance to maintain the expression of resistance in aging females, or at least to reduce the decline in resistance expression with age. Furthermore, the presence of *kdr* likely enhances this effect on the maintenance of resistance phenotype in blood-feeding females. This is suggested by the observation that the resistant phenotype produced by multiple blood meals is maintained for a prolonged period after feeding for DDT and permethrin, but the effect on deltamethrin resistance is restricted to the period immediately after feeding. This may be related to the presence of the blood-meal itself, rather than lasting metabolic changes induced by multiple feeding. However, it is more likely that variation in the relative contributions of metabolic detoxification and *kdr* to the expression of each phenotype account for these differences.

These data also suggest that *kdr* does not act independently of metabolic mechanisms in SENN-DDT. Although resistance to DDT is often primarily mediated by *kdr* in the closely related malaria vector *An. gambiae* (reviewed in [35]), the decreasing expression of the resistance phenotype with age in the control (non-blood fed) and single blood meal cohorts in this study suggests that without increased GST activity induced by blood meals *kdr* becomes less efficient with age. However, DDT resistance is never completely lost, in contrast to Cytochrome P450-mediated deltamethrin resistance, suggesting that *kdr* does play a significant role in production of the DDT resistance phenotype, but that this

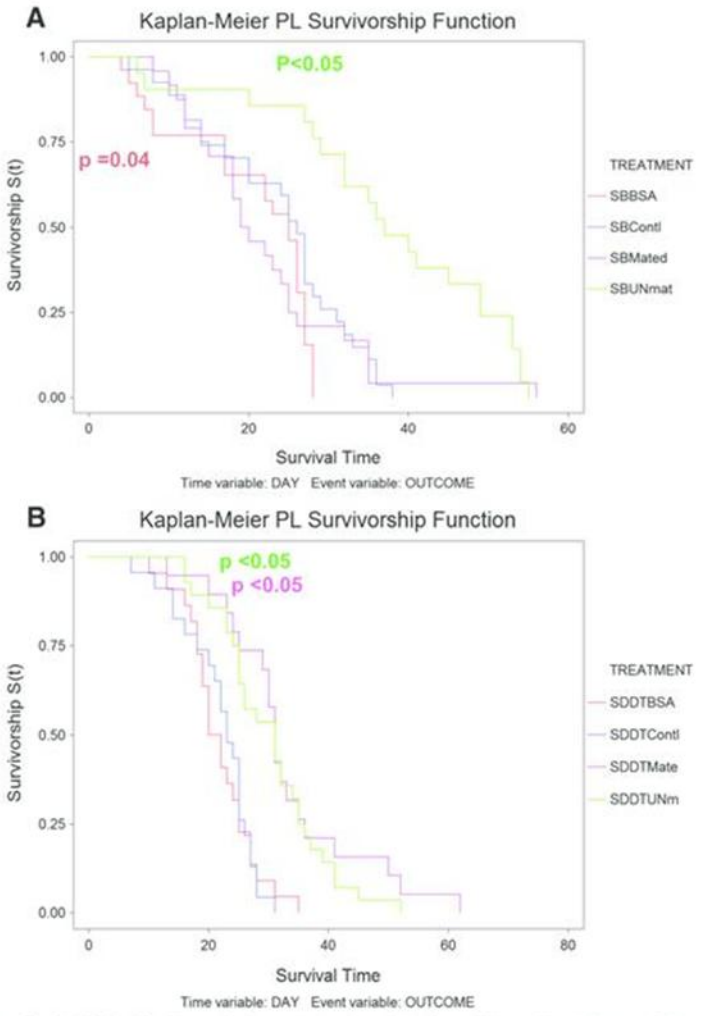


Figure 6 The effect of multiple blood-feeding on the longevity (survivorship) of *Anopheles arabiensis* SENN and SENN-DDT females (Blocks A and B respectively). Light blue and pink lines represent unmated and mated respectively. The effect of Bovine Serum Albumin (BSA) on the longevity of SENN and SENN-DDT females are also displayed, with the BSA treated females represented by the dark blue line. The longevity of all the treatments were compared to that of the sugar fed controls (red line).

phenotype is primarily mediated by metabolic detoxification in SENN-DDT.

Of particular importance in these results is the data showing that older females that had taken multiple blood meals were not necessarily more susceptible to insecticides than younger females, which has implications for any control methods designed to target older females using lower doses of insecticide [22]. Vector control efficacy cannot be restored in insecticide resistant populations using this method if older, potentially infective females are as resistant to insecticides as their younger counterparts.

This study also demonstrates the variability of detoxification enzyme expression/production with age and that a

continuous decline of enzyme activity with age is not the universal enzymatic behaviour. Multiple blood meals evidently suppress Cytochrome P450 activity, as also suggested previously by the down regulation of 22 P450 transcripts in blood fed *An. gambiae* females [38]. As cytochrome P450 activity induces oxidative stress [39,40], this down regulation is likely a mechanism to reduce basal oxidative stress in order to cope with that induced by the blood meal [38]. Blood feeding, either as a single or multiple meals, sustains a reduction in P450 activity until 21 days. Multiple blood meals are also significantly associated with elevated GST activity throughout the female's lifetime. Changes in esterase activity do not appear to correlate with any of the insecticide resistance phenotypes recorded in SENN-DDT. The increase in esterase activity in older females that had taken multiple meals may be due to reproductive functions in the female [41,42].

The basis of reduced toxicity was investigated by Halliday and Feyereisen (1987), but no conclusion could be drawn about how the effect was mediated. This study may provide some insight into this mechanism. From the biochemical analysis, it is evident that multiple blood-meals result in sustained GST activity and suppressed cytochrome P450 activity. This is a confirmation of previous microarray studies that demonstrated the reduction of transcript abundance of various cytochrome P450s, but the increase of various GST transcripts and several antioxidant enzymes in response to blood-feeding in both laboratory [27] and field caught anophelines [38]. Muller *et al.* (2008) speculate that the response observed may be the mosquito's system trying to shift towards a reduced state of oxidative stress as the activity of cytochrome P450s tend to induce oxidative stress [39,40]. As reactive oxygen species concentration in the gut is relieved by a blood-meal [43], it is possible that the modulation of oxidative stress may play an important role in the effect of blood-feeding-induced reduction of insecticide toxicity and is a factor that is worth investigating.

Anopheles gambiae females generally forego sugar feeding if possible and feed frequently and preferentially on blood [10]. This may be true for *An. arabiensis* as well. The effect of multiple blood meals on the subsequent longevity of females is important. Mated *An. gambiae* females show reduced survivorship on sugar and blood than those that have not mated [44], suggesting a reproductive cost to these females. The present study mirrored this effect in insecticide susceptible SENN females in which multiple blood feeding increased the longevity of unmated, but not mated females. These data suggest that mated females shift their resources towards egg production at the expense of longevity. Interestingly, this effect was not mirrored in resistant SENN-DDT, where multiple blood feeding increased longevity regardless of mating status.

Conclusions

In conclusion, multiple blood feeding is associated with the maintenance of DDT and pyrethroid resistance in aging *An. arabiensis* females harbouring *kdr* and metabolic resistance mechanisms. Previous studies have demonstrated a reduction in ROS after a blood-meal [43]. The effect observed in this study may be mediated by a reduction of oxidative stress in order to cope with ingested blood, which in turn reduces ROS-mediated insecticide toxicity. This effect is likely mediated by GSTs. The importance of GST-mediated defence against oxidative stress on pyrethroid toxicity has already been demonstrated [45]. Further investigations into these assertions are in progress (unpublished data). Multiple blood meals also favour survivorship of insecticide resistant females. Multiple blood-feeding is a common occurrence in anopheline mosquitoes. This does not only have reproductive and epidemiological consequences, but may also affect insecticide based malaria vector control measures directed against insecticide resistant vector populations.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SVO contributed to the experimental design, performed the experiments, did the original data analysis and produced initial drafts of the manuscript. BDB conceived the project, assisted with data analysis and produced the final version of the manuscript. Both authors read and approved the final version of the manuscript.

Acknowledgements

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Appendix 2: Ethical approval

M130534

R14/49 Ms Shune Oliver

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130534

NAME: (Principal Investigator)	Ms Shune Oliver
DEPARTMENT:	School of Pathology/MERU National Institute for Communicable Diseases
PROJECT TITLE:	The Effect of environmental Stress on the Life Hisotry and Insecticide Phenotypes of African Anopheline Malaria Vectors
DATE CONSIDERED:	31/05/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Basil Brooke
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 31/05/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix 3

Specialised reagents and equipment

Assay	Equipment Item	Supplier
Hydrolysis probe analyis	C-1000Thermal cycler with CFX96 real time system	Bio-Rad
Biochemistry Assay	Multiskan ascent	Thermo Scientific

Assay	Reagent	Supplier	Catalogue number
Detoxification enzyme assays	Bradford Reagent	Bio-Rad	500-0006
	DTNB	Sigma Aldrich	D8130
	CDNB	Sigma Aldrich	237329
	α-naphthyl acetate	Sigma Aldrich	N8505
	β-napthyl acetate	Sigma Aldrich	N6875
	α – naphtol	Sigma Aldrich	N-1000
	β- naphtol	Sigma Aldrich	N-1250
	Cytochrome C	Sigma Aldrich	C-3006
	Reduced gultathione	Sigma Aldrich	G4251
	Fast Blue	Sigma Aldrich	F0500
Oxidative stress enzyme assays	Oxidised glutathione	Sigma Aldrich	G4376
	Glutathione reductase	Sigma Aldrich	
	NADPH	Sigma Aldrich	N9660
	DNPH		42210
	Cumene Hydroperoxide		247502
	TCA	Sigma Aldrich	T6399
	Guanidine hydrochloride		G4505
Insecticides and Synergists	DDT	Sigma Aldrich	49019
	TPP	Sigma Aldrich	241288
	PBO	Sigma Aldrich	291102
	DEM	Sigma Aldrich	D97703
	ATZ	Sigma Aldrich	A3506
	DDC	Sigma Aldrich	D35056
	Haematin	Sigma Aldrich	H3281
Miscellaneous	Human insulin	Sigma Aldrich	I2643
	Quercertin	Sigma Aldrich	Q4951
	WHO Bioassay Papers	School of Biological Sciences, Universiti Sains Malaysia (USM), Penang, Malaysia	

Appendix 4: Protocol for preparation of synergists

a) SOP: Preparation of synergist papers
b) Department: Vector Control Reference Laboratory
c) Date: 3 October
d) SOP written by: Shüné Oliver

1. **The purpose of the method (identifies what the method is used for).**

To prepare synergist papers to be used in synergist bioassays

2. **The source of the method and/or literature reference.**

Procedures for Diethyl maleate and Triphenyl phosphate optimised in-house by Shüné Oliver

Bernard CB, Philogène BJ. **Insecticide synergists: role, importance, and perspectives.** J Toxicol Environ Health. 1993 Feb;38(2):199-223.

Oppenoorth FJ. **Resistance in insects: the role of metabolism and the possible use of synergists.** Bull World Health Organ. 1971;44(1-3):195-202.

3. **The principle of the method.**

Synergists block metabolic pathways that would otherwise be involved in conferring resistance to insecticides. A synergist-induced change in resistance phenotype would implicate a metabolic pathway as the mode of resistance.

4. **Identification of potentially hazardous procedures/operations/ substance where these may occur in the method.**

All synergists used are potentially harmful as they block the enzyme pathways of all eukaryotes.

5. **The nature and volume of specimen required.**

The protocol is a preparative step and as such does not require a specimen.

6. Equipment and reagents required.

Piperonyl butoxide

Diethyl maleate

Triphenyl phosphate

Acetone

Chloroform

Olive oil

Filter paper squares (15X11.5cm squares, whatman no 1)

Micropipettes capable of aliquotting 200 and 1000µl.

7. The preparation and storage of reagents.

The stock solutions are prepared as follows

Piperonyl butoxide (4% stock)

- 4.4 ml of 90% piperonyl butoxide concentrate in 95.6 ml of olive-oil acetone mixture (1:1).

Diethyl maleate (20% stock)

- 4.1 ml 97% diethyl maleate concentrate in 15.9ml of olive-oil acetone mixture (1:1)

Triphenyl phosphate (20% Stock)

- 5g triphenyl phosphate in 10 ml chloroform
- Add 15 ml of olive-oil acetone mixture (1:1) to triphenyl phosphate/chloroform solution

All sock reagents can be stored at room temperature indefinitely. Prepared papers can be stored in foil at 4^{o}C for several months.

8. Detailed step-by-step instructions on how to perform the test

1. Prepare filter paper: Watman no 1, 11.5 X 15cm labelled with synergist, concentration and date, written in pencil.
2. For each paper mix 0.7ml stock solution and 1.2 ml olive oil-acetone (carrier solution).
3. Spot the total 1.9ml solution onto paper until the page is saturated. Spot onto the side of the paper not labelled in pencil.
4. Dry overnight
5. Place into WHO bioassay tube with writing facing outward.
6. Test paper on susceptible colony, as per insecticide paper testing protocol

Appendix 5: Formulae for enzyme calculations

1. *Calculation of GST activity:*

ΔA340 min-1 x 0.2 mL Reaction Volume

0.0096 μmol-1 cm-1 x 1000 mL x 0.524 cm x A

= 0.0396 x A340 min-1 x Sample Dilution/A (μmol/min/mL)

Where: 0.0096 μM-1 cm-1 is GS-DNB extinction coefficient.

A is sample volume in the reaction well in milliliter (mL).

0.524 cm is light path of the 0.2 mL Reaction Volume in 96 well plate (cm).

2. *Calculation of Protein Carbonyl Content*

Carbonyl Content (nmol.ml)= [(Corrected absorbance)/0.011μM-1)](500μl/200μl)

Where corrected absorbance is average absorbance of untreated control substracted from the treated samples.

3. *Calculation of Catalase activity*

$$\text{Activity} = \frac{\Delta\mu\text{mol (hydrogen peroxide)} \times D}{V \times T}$$

Where: Δμmol (hydrogen peroxide) is amount of hydrogen peroxide present corrected for with a blank value

D= Dilution of original sample

T= reaction duration in minutes

V= sample volume

4. *Calculation of Glutathione peroxidase activity:*

ΔA_{340}/min= A340 (Time 2)-A340 (Time 1)

Time 2(in minutes)-Time 1(in minutes)

These two timepoints are defined as the beginning and the end of the linear phase of the enzyme reaction

GPOX activity = A340/min x total volume x sample dilution= nmol/min/ml

0.00373µM-1* sample volume

*= extinction co-efficient adjusted for the pathlength of the 96-well plate

Appendix 6: Uniprot Data

Uniprot Results for reported proteins in *Anopheles gambiae*: Catalase
(http://www.uniprot.org/uniprot/?query=organism%3A*Anopheles*+gambiae+catalase&sort=score)

	Entry	Entry name	Protein names	Gene names	Organism	Length	e
☐	A3E7Z8	A3E7Z8_ANOGA	Catalase (EC 1.11.1.6)	**CAT1**, AgaP_AGAP004904	*Anopheles* gambiae (African malaria mosquito)	504	
☐	Q6RBZ5	Q6RBZ5_ANOGA	Catalase (EC 1.11.1.6)		*Anopheles* gambiae (African malaria mosquito)	485	
☐	A3E807	A3E807_ANOGA	Catalase (EC 1.11.1.6)		*Anopheles* gambiae (African malaria mosquito)	504	
☐	A3E800	A3E800_ANOGA	Catalase (EC 1.11.1.6)	**Cat**	*Anopheles* gambiae (African malaria mosquito)	504	

Uniprot Results for reported proteins in *Anopheles gambiae*: Superoxide Dismutase (http://www.uniprot.org/uniprot/?query=organism%3A*Anopheles*+gambiae+superoxide+dismutase&sort=score)

Entry	Entry name	Protein names	Gene names	Organism	Length	e
Q7QDP9	Q7QDP9_ANOGA	**Superoxide dismutase [Cu-Zn]**	**CUSOD3**, AgaP_AGAP010347	*Anopheles* gambiae (African malaria mosquito)	153	
A7UTA9	A7UTA9_ANOGA	**Superoxide dismutase [Cu-Zn]**	**CUSOD2**, AgaP_AGAP005234	*Anopheles* gambiae (African malaria mosquito)	171	
Q7Q9H5	Q7Q9H5_ANOGA	**Superoxide dismutase [Cu-Zn]**	**CUSOD2**, AgaP_AGAP005234	*Anopheles* gambiae (African malaria mosquito)	207	
Q7Q8W6	Q7Q8W6_ANOGA	**Superoxide dismutase**	**MNSOD1**, AgaP_AGAP010517	*Anopheles* gambiae (African malaria mosquito)	219	
Q6QVQ5	Q6QVQ5_ANOGA	**Superoxide dismutase [Cu-Zn]**	**SOD2**	*Anopheles* gambiae (African malaria mosquito)	211	
Q5XNS4	Q5XNS4_ANOGA	**Superoxide dismutase [Cu-Zn]**	**SOD3**	*Anopheles* gambiae (African malaria mosquito)	75	
Q6RBZ4	Q6RBZ4_ANOGA	**Superoxide dismutase 1**	**SOD1**	*Anopheles* gambiae (African malaria mosquito)	206	

Appendix 6: Summary of strain information

Strain	Established	Origin	Species	Resistance phenotype	Mechanism
SENN	1980	Sennar, Sudan	*An. arabiensis*	Baseline resistance	N/A
SENN DDT	1995	Sennar, Sudan	*An. arabiensis*	DDT, pyrethroid, malathion resistance	metabolic, kdr
KGB	1970s	Kanyembe, Zimbabwe	*An. arabiensis*	Fully susceptible	N/A
FANG	2000s	Angola	*An funestus*	Fully susceptible	N/A
FUMOZ	2000s	Mozambique	*An funestus*	Baseline resistance	Metabolic: P450
FUMOZ R	2000s	Mozambique	*An funestus*	Pyrethroid, Bendiocarb resistance	Metabolic: P450
SUA	1986	Suakoke, Liberia	*An. coluzzi*	Fully susceptible	N/A
ZAMG	Wild-2013 collection	Zambia	*An. gambiae*	Pyrethroid resistance	Metabolic: P450
CLADE I	Wild-2013 collection	Zambia	*An. funestus*	Pyrethroid, Bendiocarb resistance	Metabolic: P450
CLADE II	Wild-2013 collection	Zambia	*An. funestus*	Pyrethroid, Bendiocarb resistance	Metabolic: P450