RESPONSES TO PYRETHROIDS IN HEAVY METAL TOLERANT *ANOPHELES GAMBIAE* VECTOR OF MALARIA

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DECLARATION AND RECOMMENDATION

Declaration

I declare that this thesis is my original work and has not been submitted for award in any institution of learning.

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We confirm that this thesis has our approval to be presented for examination as per the Egerton University regulations.

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DEDICATION

To my beloved mother Maimuna Abdallah, whose contribution towards my education is priceless, and to my dearest friend, the Late Ibrahim Said Rajab.

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ABSTRACT

Anopheles gambiae mosquito, the principle Afro-tropical vector of malaria, has been documented in habitats with heavy metals in excess of natural loads. The mosquito has also displayed resistance to most conventional insecticides, and potential to resist challenges by heavy metals. Investigations were conducted in strains of *An. gambiae* ss, in which tolerance to cadmium and lead heavy metals were established. This study was undertaken to determine any putative relationship between tolerance to (cadmium and lead) heavy metals, and resistance to pyrethroid insecticide in *An. gambiae*. Tolerance to cadmium and lead heavy metals was developed in larval stages and this development in tolerance was monitored using changes in lethal concentration $(LC)_{50}$ of the individual metals between successive generations using bioassay technique. Heavy metal tolerant males and females individuals and relevant controls were separately screened for 1) expression of pyrethroid metabolic CYP6M2, CYP6P3 and CYP6Z1 genes of cytochrome p450 enzyme by quantitative PCR (qPCR) and 2) knockdown resistance gene mutation (*kdr*) conferring resistance to pyrethroids through standard PCR and sequencing. Phenotypic responses of the mosquitoes to pyrethroid insecticides were assessed by bioassay according to the standard WHO procedure for monitoring resistance to insecticides. Differences in expressions of the cytochrome p450 genes among the populations was determined by Analysis of Variance (ANOVA), and assessment of leucine to serine substitution that confer *kdr* gene mutation was conducted by multiple sequence alignment. The *kdr* mutation was not detected among the metal tolerant populations, and the LC_{50} responses to permethrin among the respective metal tolerant and control mosquito populations were similar. However, expressions of all the classes of cytochrome p450 genes were significantly lower in females than males ($F_{(1,\ 46)}$=30.13 , $p<0.0001$) irrespective of treatment. Within gender, the reductions in expression were more pronounced in the male metal tolerant, relative to their respective controls populations. There were general reductions in expression levels of all the genes in metal tolerant relative to control population, where significantly lower expressions ($F_{(2,\ 46)}$= 7.06, p= 0.0021) of the all genes were observed in the metal tolerant than in control male populations. The expression of all the genes in cadmium selected female populations was higher than in their respective controls, although the difference was not statistically significant ($P > 0.05$). These results suggest that adaptation to cadmium and lead heavy metals does not confer resistance to pyrethroid insecticides. Thus urban mosquitoes breeding in heavy metal polluted waters are susceptible to these insecticides, as long as the habitat is not compromised by any other sources of contamination from urban pollution. The understanding of the mechanism that results to emerging pyrethroid resistance is very important, since it will shade light on the necessary improvement strategies that are required to enhance its effectivity and thus ensure eradication of the vector and control of the disease.

TABLE OF CONTENTS

DECLARATION AND RECOMMENDATION ii
COPYRIGHT iii
DEDICATION iv
ACKNOWLEDGEMENT v
ABSTRACT vi
TABLE OF CONTENTS vii
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF ABBREVIATIONS xi
CHAPTER ONE 1
INTRODUCTION 1
1.1 Background information 1
1.2 Statement of the problem 2
1.3 Objectives 2
1.3.1 General objective 2
1.3.2 Specific objectives 2
1.4 Hypotheses 3
1.5 Justification 3
CHAPTER TWO 4
LITERATURE REVIEW 4
2.1 Introduction to malaria and urbanisation 4
2.2 Evolutionary history of *An. gambiae* 5
2.3 Heavy metals 6
2.3.1 Effect of heavy metals on *An. gambiae* 6
2.3.2 Mechanism of heavy metal resistance 8
2.4 Conventional insecticides 9
2.4.1 Classification of insecticides 9
2.4.2 Mechanism of action of insecticides 10
2.4.3 Mechanism of insecticide resistance 11
2.4.4 Methods of detecting insecticide resistance 12
CHAPTER THREE 14
MATERIALS AND METHODS 14

3.1 Selection for tolerance to cadmium and lead in *An. gambiae* ss 14
3.2 Extraction of RNA and DNA from adult *An. gambiae* ss.....14
3.3 Simultaneous species verification and molecular form characterization.....15
3.4 Quantification of cytochrome p450 genes 15
3.4.1 Reverse transcriptions 15
3.4.2 Real-time qRT PCR of CYP6Z1, CYP6P3 and CYP6M2 CYP6 P450 enzymes.....16
3.5 Detection of *kdr* mutation by PCR amplification sequencing 18
3.6 Bioassay for monitoring resistance.....20
3.7 Data analysis 20
CHAPTER FOUR.....21
4.1 Results.....21
4.1.1 Expression profiles of metal responsive classes of CYP6 p450 genes 21
4.1.2 Molecular forms and kdr genotypes in cadmium and lead tolerant *An. gambiae*.....24
4.1.3 Phenotypic behaviour of *An. gambiae* on exposure to insecticide papers 24
4.2 Discussion.....26
CHAPTER FIVE 28
CONCLUSION AND RECOMMENDATION 28
5.1 Conclusion 28
5.2 Recommendation 28
CHAPTER SIX 30
REFERENCES.....30
APPENDICES.....37

LIST OF TABLES

Table 1:Primers used for quantitative PCR studies 17

Table 2: Primer used for detection of pyrethroid kdr resistance or susceptibility mutation ..19

Table 3:. Relative mean (± SE) expression levels of various classes of cytochrome p450 genes in heavy metal tolerant adult An. gambiae s. s mosquito.....23

Table 4: Yield and quality of extracted RNA of adult *An. gambiae* ss mosquito determined by spectroscopy.....42

Table 5: Analysis of variance results of the effect of the gene, gender, treatment and gender treatment interaction factors on the linear model.....43

Table 6: Parameter estimates and their associated p-values, showing pair-wise mean comparison within gene, gender and treatment factors.....44

Table 7: Detailed pair-wise mean comparison of gender-treatment interaction within the various treatments.....45

LIST OF FIGURES

Figure 1: Sex specific influence of cadmium or lead tolerance on expressions of cytochrome p450 genes associated with pyrethroid resistance in *An. gambiae* ss mosquito vector of malaria.....22

Figure 2: Molecular forms, knockdown resistance alleles (*kdr*) and associated mutations in cadmium or lead tolerant *An. gambiae* ss.....25

LIST OF ABBREVIATIONS

ACUC	Animal Care and Use Committee
ARCU	Animal Rearing and Containment Unit
AP-1 DNA	Activator Protein-1 Deoxyribonucleic Acid
Ct	Crossing threshold
kdr	Knockdown Resistance
LC_{50}	Median Lethal Concentrations
MATCs	Maximum Acceptable Toxicant Concentrations
MRE	Metal Responsive Element
PKC	Protein Kinase C
SNP	Single Nucleotide Polymorphism

CHAPTER ONE
INTRODUCTION

1.1 Background information

Anopheles gambiae mosquito is expanding its ecological niche into polluted habitats in urban areas. Larvae of the mosquito thrive in water bodies polluted with domestic and/or industrial sewage (Awolola *et al*., 2007; Djouaka *et al*., 2007), including heavy metals in excess of natural loads in urban areas (Mireji *et al*., 2008). Coincidentally, pyrethroid insecticides have also been extensively used in the urban areas to control the vectors, exerting additional selection pressure on the mosquito that has resulted in development of resistance in the vector to the insecticides (Elissa *et al.,* 1993; Vulule *et al.,* 1994). The pyrethroid insecticide resistance has been documented in Sub-Saharan Africa, and in Western Kenya whereby the frequency of resistance allele was seen to increase after insecticide application (Stump *et al.,* 2004). The resistance has in turn facilitated colonization by the mosquitoes of potentially polluted habitats unfavorable to susceptible predators and competitors of the mosquitoes (Chandre *et al*., 1999; Diabaté *et al*., 2002; Diabaté *et al*., 2004; Chouaïbou *et al*., 2008). Implications of the adaptation of the mosquitoes to the heavy metals on the observed resistance to the pyrethroids has not been determined, and is critical in understanding how the heavy metal tolerance can potentially pre-dispose the mosquito to resistance to the insecticides.

In a previous study it was demonstrated that the adaptation to heavy metals potentially results in significant loss in biological fitness in the mosquito (Mireji *et al.,* 2010a), through a process that is accompanied by induction or expression of a spectrum of potential metal responsive proteins (Mireji *et al.,* 2006) including metallothionein and alpha-tubulin (Mireji *et al.,* 2010b). Studies elsewhere have also established that classes within CYP6 superfamily of cytochrome p450 xenobiotic responsive genes: CYP6M2, CYP6P3 and CYP6Z1, directly and metabolicaly counteract insecticidal effects of pyrethroinds in *An. gambiae* (Nikou *et al*., 2003; Djouaka *et al*., 2008 and Muller *et al*., 2008). Rapid expansion and diversification of these enzymes in the mosquito have also adapted them to counteract anthropogenic heavy metal xenobiotics (Poupardin et al., 2008). The metals have also been implicated in induction of cellular damage through oxidative stress that culminate in intra-chromosomal DNA damage (Stohs *et al.,* 2001)

and delay or inhibit DNA synthesis and repair respectively (Enger *et al.,* 1987; Hartwig *et al.,* 1996), leading to genetic mutations.

Pyrethroid resistance has coincidentaly been associated with *kdr* mutations, a leucine substitution at position 1014 of the voltage-gated sodium channel (Martinez-Torres *et al.,* 1998). The leucine is either substituted by phenylalanine or serine in West or East Africa respectively (Ranson *et al.,* 2000). Information on influence of heavy metal tolerance on expression of the CYP6 genes or *kdr* mutations, and subsequent succesptibility of *An. gambiae* ss to pyrethroids can shed light on contribution of pollution of habitats of the mosquito with heavy metals on modulation of susceptibility/resistance of the moquito to pyrethroid insecticides.

1.2 Statement of the problem

An. gambiae ss, the vector of malaria has adapted to heavy metal pollutants in their natural habitats. In addition, the mosquito has developed resistance to most of the conventional insecticides employed in the vector control initiatives. However, influence of the vector to heavy metal adaptation in their natural habitats on their subsequent responses to conventional insecticides, and possible molecular/genetic factors associated with the responses have not been established. This will be important in modification of the pyrethroid insecticide in order to increase its effectivity, and consequently facilitating the vector eradication and disease control.

1.3 Objectives

1.3.1 General objective

To determine the genetic and phenotypic pyrethroid associated responses in *An gambiae* ss tolerant to selected heavy metals.

1.3.2 Specific objectives

1. To determine differential expression profiles of pyrethroid responsive cytochrome p450 enzymes in cadmium and lead tolerant *An. gambiae* ss.
2. To determine presence of *kdr* mutation conferring pyrethroid resistance in cadmium and lead tolerant *An. gambiae* ss.

3. To determine differential susceptibility of cadmium or lead heavy metal tolerant *An. gambiae* ss mosquito to pyrethroid insecticides.

1.4 Hypotheses

1. Cadmium or lead tolerance in *An. gambiae* ss. does not influence differential expression profiles of pyrethroid responsive cytochrome p450 enzymes in the mosquito.
2. Cadmium or lead tolerance in *An. gambiae* ss. do not influence occurrence of *kdr* mutation conferring pyrethroid resistance in the mosquito.
3. Cadmium or lead tolerance in *An. gambiae* ss does not induce differential susceptibility of the mosquito to pyrethroids insecticides.

1.5 Justification

An. gambiae ss. appears to have expanded its niche to polluted habitats replete with xenobiotics, including heavy metals and pyrethroid insecticides. The heavy metals are in excess of natural loads and are from anthropogenic origins such as industrial pollution, where as the pyrethroid insecticides are anthropogenic from routine domestic insect pest control and agricultural applications. While concurrent presence of heavy metals and pyrethroids in the natural aquatic habitats of the mosquito have been documented, potential pre-disposition for subsequent pyrethroids resistance/susceptibility in the mosquito by the metals has not been determined. Additionally, common underlying molecular/ genetic mechanisms influencing proliferations of the mosquitoes in the heavy metal and insecticide polluted habitats have not been determined.

It is therefore necessary to assess these parameters to shed light on the potential contribution of heavy metals tolerance in the mosquito in the emergence of insecticide resistance/susceptibility observed in *An. gambiae* in nature. This will facilitate any possible recommended modifications on the pyrethroid insecticide in order to increase its efficacy and thus facilitate vector eradication and malaria disease control.

CHAPTER TWO
LITERATURE REVIEW

2.1 Introduction to malaria and urbanisation

Malaria has been considered a predominantly rural disease in Africa primarily because suitable proliferation sites for *Anophelines* are scarce in highly populated and polluted urban areas (Robert *et al*., 2003; Donnelly *et al*., 2005; Hay *et al*., 2005). The process of urbanization and increasing human densities has eliminated open spaces for mosquito breeding and increased pollution of the remaining breeding sites, limiting opportunities for vector development and survival (Trape and Zoulani, 1987) Malaria exposure per capita has decreased with increased human densities as a consequence of the scarcity of suitable and productive larval development sites (Robert *et al*., 2003).

Adaptation of malaria vector mosquitoes to the urban environment in Africa can have dramatic consequences for the epidemiology of this devastating disease, as the urban population in Africa is rapidly increasing. Urban dwellers exceeded the rural population in 2008 for the first time in history and by 2030, 54% of Africans are expected to live in urban areas with an estimated 0.9 billion additional urban dwellers in Africa by 2050 (UN, 2008). A specific informal sector is urban agriculture, which has been recognized as a means of increasing food supply in the growing cities and contributing to improved nutrition, employment and poverty alleviation (Afrane *et al*., 2004). However, the immediate benefits of increasing intensive urban agriculture may have some drawbacks for human health and the environment, mainly through high pesticides and /or fertilizer inputs use with some crops regarded as sources of pollution (Atis, 2006).

Modified urban settings and resulting landscape alterations creates “rural islands” within cities, which provide suitable breeding sites for mosquitoes and result in invasion of urban environment by malaria vectors (Dossou-yovo *et al*., 1994; Robert *et al*., 1998; Antonio-Nkondjio *et al*., 2005). Water bodies in such areas are likely to carry heavy loads of nutrients, organic matter and heavy metals, in addition to other toxicants from municipal, industrial and agricultural sources. These create selective pressure for tolerance to pollutants in the mosquito. A number of recent studies have reported observation of *An. gambiae* larvae thriving in a variety of anthropogenic urban water collections, some of which were highly polluted. For example, drains containing

domestic wastewater or oil spillage from refineries and sewage ponds with organic pollution from human feces in Dar es Salaam, Tanzania (Sattler *et al*., 2005), Lagos and Ibadan, Nigeria (Awolola *et al*., 2007; Djouaka *et al*., 2007), Accra and Tema, Ghana (Chinery, 1984), Kinshasa, Democratic Republic of Congo (Coene, 1993) or Kisumu and Malindi, Kenya (Mireji *et al*., 2008).

2.2 Evolutionary history of *An. gambiae*

There are currently seven recognized sibling species in the *An. gambiae* complex, including *An. gambiae* ss. and *Anopheles arabiensis*, which are the most efficient and widespread vectors of malaria in sub-Saharan Africa (Gillies and de Meillon, 1968). Both species are highly anthropophilic with their larvae typically developing in structurally simple, temporary water collections deprived of vegetation and strongly exposed to the sun (Gillies and de Meillon, 1968). Several lines of evidence suggest that *An. arabiensis* might be the ancestral species of the complex (Ayala and Coluzzi, 2005; Sharakhova *et al*., 2006). Originally zoophilic and exophilic, *An. arabiensis* may have first dispersed in East Africa more than 6,000 years ago, where it gradually became anthropophilic as human population densities increased (Willis *et al*., 2004).

The origin of the highly anthropophilic *An. gambiae* can be traced back to the late Neolithic age before 4,000 BC when the first traces of human agricultural activities were recorded in the impenetrable African rainforest belt (Willis *et al*., 2004). Extensive penetration of the forest began about 3,000 BC., aided by climate change and the temporary "savannization" of much of the central African rain forest that lasted about 5 centuries (Willis *et al*., 2004). When the forest regained its original range of distribution about 2,300 BC., it was invaded by Bantu agriculturalists who adopted "slash-and-burn" agricultural techniques, therefore clearing the soil from humus which prevented accumulation of temporary water bodies. The clearing enhanced the opening of the forest canopy and provided ample opportunities for *An. gambiae* breeding in temporary sunlit pools (Besansky *et al*., 2003; Sharakhova *et al*., 2006). These conditions promoted strong selection for anthropophily and domesticity in *An. gambiae*, creating one of the most powerful vector systems for the malaria parasite *Plasmodium falciparum*. Spreading of this highly anthropophilic *An. gambiae* from the rainforest into savanna areas was probably achieved also through close association with humans and secondary contacts with the savanna-adapted *An.*

arabiensis from which it acquired chromosomal inversions allowing adaptation to more arid environments through genetic introgression (Besansky *et al*., 2003; Sharakhova *et al*., 2006). Further speciation is still ongoing in *An. gambiae*, as evidenced by genetic discontinuities revealed through study of distribution of chromosomal and molecular polymorphisms within and between natural populations of the species (Coluzzi *et al*., 1979; Favia *et al*., 2001; della Torre *et al*., 2002; della Torre *et al*., 2005). Of particular relevance is the description of the Mopti chromosomal form, which has evolved the ability to proliferate in permanent, man-made water reservoirs and irrigation schemes in arid areas (Touré *et al*., 1994). There is an almost exact correspondence between molecular and chromosomal forms in the savannah areas of West Africa (della Torre *et al*., 2002; della Torre *et al*., 2005).

The most recent, and maybe the most striking example of the influence of human agricultural practices on the evolutionary history of *An. gambiae*, comes from the study of insecticide resistance. In rural areas of West Africa, there is evidence that the widespread use of insecticides for agricultural purposes, especially for cotton crop protection, has favored emergence and subsequent spread of insecticide resistance in malaria vectors (Chandre *et al*., 1999; Diabaté *et al*., 2002). This set of evidences demonstrates the remarkable evolutive advantages of *An. gambiae sensu lato* to colonize new environments that were not conducive to its ecological optimum. The same may probably apply currently under urban settings, with the dramatic consequence of favoring adaptation of this pest species to urban environment, by promoting tolerance to urban xenobiotics which include heavy metals (Diabaté *et al*., 2004; Chouaïbou *et al*., 2008).

2.3 Heavy metals

2.3.1 Effect of heavy metals on *An. gambiae*

The heavy metals can affect many cellular functions by modification of cell growth and metabolism at sub-lethal and marginally lethal levels, and apoptosis and necrosis at lethal levels. Heavy metals also exert effects with potential genetic consequences by inhibiting; cellular DNA repair mechanisms (Hartwig *et al.*, 1996), DNA polymerases and RNA transcription and translation enzymes and proteins (Beyersmann and Hechtenberg, 1997).

It is hypothesized that cadmium induces cellular damage through oxidative stress (Stohs *et al.*, 2001) by binding to reduced cystein residues and generating reactive oxygen species and by lowering intracellular glutathione levels (Manca *et al.,* 1991; Chin and Templeton, 1993). The reactive oxygen species are mainly composed of superoxide ion, hydroxyl radicals, and hydrogen peroxide. The oxidative stress is accomplished through lipid peroxidation, modulation of intracellular oxidized states, production of nuclear factor-kappaB and induction of DNA damage. In addition, cadmium increases lipid peroxidation and other oxidative stress factors by inhibiting glutathione reductase (Koizumi and Li, 1992; Acan and Tezcan, 1995) and superoxide dismutase (Hussain *et al.,* 1987), reducing catalase, increasing glucose-6-phosphate dehydrogenase and glutathione peroxidase activities (Salovsky *et al.,* 1992). Moreover, these reactive oxygen species also induce membrane damage, alter gene expression, induce apoptosis and delay DNA synthesis (Enger *et al.,* 1987). They also disrupt intracellular sulfhydryl homeostasis (Beyersmann and Hechtenberg, 1997) and induce tumors (Abshire *et al.,* 1996).

Heavy metals such as cadmium, evoke intracellular effects via a reversible interaction with an external receptor on the cell surface as observed in human skin fibroblasts (Smith *et al.,* 1989). They can also enter through receptor operated calcium channels (Blazka and Shaikh, 1991), passive transport (Shaikh *et al.,* 1995), SH-ligand containing transport processes associated with the uptake of zinc, copper and iron and voltage-sensitive calcium channels (Hinkle and Osborne, 1994). The metals modulate activities of complex signal transduction pathways that in turn influence expression of a myriad of genes. The pathways include those mediated by Protein Kinase C (PKC), calcium or zinc cAMP dependent protein kinases, calmodulin and mitogen activated protein kinases (Goldstein, 1993; Beyersmann and Hechtenberg, 1997; Templeton *et al.,* 1998; Bressler *et al.,* 1999; Son *et al.,* 2001).

The metals activate calmodulin-mediated pathway by substitution of calcium in binding to calcium binding sites (Goldstein and Ar, 1983; Behra and Gall, 1991). Lead has also been shown to improperly substitute for zinc in aminolevulinic acid dehydrogenase, (Warren *et al.,* 1998). Carbonic anhydrase, a zinc dependent enzyme is also inhibited by lead (Goering, 1993). Although lead is effectively able to compete with zinc to bind to zinc sites, lead does not induce the proper structural conformation once bound (Hanas *et al.,* 1999), leading to aberrant

expression of the *Sp1* target gene such as myelin basic protein and proteolipid protein (Zawia and Harry, 1996).

Protein Kinase C (PKC) activation may also be induced through the cadmium-induced oxidative stress (Bagchi *et al.,* 1997; Stohs *et al.,* 2001). Upon activation by cadmium, PKC has been observed to mediate induction of early response genes (Tang and Enger, 1993; Matsuoka and Call, 1995; Templeton *et al.,* 1998). Induction of these proto-oncogenes is largely independent of mitogen activated protein kinase system (Beyersmann and Hechtenberg, 1997). Lead has also been observed to enhance AP-1 DNA binding through a PKC mediated pathway and induction of immediate early genes expression through activation of PKC (Kim *et al.,* 2000). Cadmium may also activate transcription through specific metal-responsive upstream regulatory elements found in the promoter regions of cadmium responsive genes, including the Metal Responsive Element (MRE) sequences found in most metallothionein genes (Stuart *et al.,* 1984).

2.3.2 Mechanism of heavy metal resistance

Experimental investigations have demonstrated the development of tolerance to increasing heavy metal challenge in *An. gambiae* ss. to cadmium, copper and lead over few generations in the laboratory (Mireji *et al*., 2006). Cells counteract the damage induced by heavy metals by transcription of genes encoding for defense and repair proteins. These proteins chelate the metal to prevent further damage, remove reactive oxygen species, repair membranes and damaged DNA and re-nature or degrade unfolded proteins (Liao and Freedman, 1998). The proteins include heat shock proteins (Abe *et al.,* 1994) and metallothionein (Shimizu *et al.,* 1997). The cells also activate proto-oncogenes, responsible for cell proliferation and/or apoptosis, by transcriptional activation of early response genes including - *c-jun, c-fos, c-myc* and *erg-1* in various cells (Jin and Ringertz, 1990; Hechtenberg *et al.,* 1996; Wang and Templeton, 1998). In case of cytotoxicity, apoptosis is induced as mode of elimination of damaged cells. Some heavy metals elicit anti-tumor effect (Terracio and Nachtigal, 1988) by induction of the p53 tumor suppressor gene (Stohs *et al.,* 2001). Some paradoxically inhibit and stimulate early and late epidermal growth factor-induced DNA synthesis (Tang and Enger, 1993).

Glutathione is an effective oxygen radical scavenger (Yu, 1994) and provides the first line of defense against some heavy metals before metallotheonein induction (Singhal *et al.,* 1987) or apoptosis (Son *et al.,* 2001). Glutathione has also been implicated in acquired cadmium resistance in human lung carcinoma A549 cells attributed to enhanced gamma-glutamylcysteine synthetase expression (Hatcher *et al.,* 1995). Metallotheonein and α-tubulin induction in *Drosophila melanogaster* larvae and *Chironomus tentans* midgut epithelia, respectively, have also been observed upon exposure to sub-lethal cadmium and copper concentrations (Maroni and Watson, 1985; Mattingly *et al.,* 2001) and may be involved in counteracting toxicity. Heavy metals can exert their toxicity to mosquitoes by displacing essential metals of a lower binding power in biologically active molecules or by acting as non-competitive inhibitors of enzymes. Conversely, mosquito populations may adapt to the heavy metal contaminants probably through development of physiological or molecular mechanisms that counteract the toxicity.

2.4 Conventional insecticides

2.4.1 Classification of insecticides

Insecticides are classified according to their chemical nature and origins. Organochloride insecticides include DDT, hexachlorohexane, cyclodienes and are most widely used. DDT was originaly considered a magic bullet in malaria control, with WHO assembly in 1955 voting for eradication of malaria following success the insecticides had achieved in reducing malaria cases in many parts of the world (WHO, 1984). The use of DDT steadily decreased because of its persistence in environment and development of resistance to it in the mosquitoes (WHO, 1994). Organophosphates insecticides such as malathion, originally used for agricultural purposes, were adopted after vector resistance to organochlorides. The malathion has low mammalian toxicity (Eto, 1974). Carbamates insecticides such as carbaryl and propoxur share mode of action with organophosphates (WHO, 1994).

Pyrethroid insecticides, constitute a new generation of highly potent synthetic insecticide derived from pyrethrins, extracted from the flower-heads of certain *Chrysanthemum* species mainly *Chrysanthemum cinerariaefolium,* grown commercially in part of Asia and Africa. From pyrethrum, a number of synthetic pyrethroids, photostable analogues such as permethrin, cypermethrin, deltamethrin and lambdacyhalothrin amongst others have been developed (WHO,

1994). Pyrethroids provides the highest selective insect toxicity compared with mammal toxicity and are easily biodegraded to a harmless product. They also have low volatility and low polarity thus restrict their movement in the air or soil from the site of application (Elliot, 1989).

Biopesticides, were developed due to the emergence and spread of insecticide resistance in many species of vectors and occurrence of multiple resistance to the other classes of insecticides in several insects. The bacterium *Bacillus thuringiensis* H-14 and *B. sphaericus* produce proteins that are toxic to mosquito and black flies larvae. The target site of the toxins is larval midgut cells where it causes degradation and lysis. Thus, the larvae undergo tremors, become sluggish, and eventually die (Rodcharoen and Mulla, 1994).

2.4.2 Mechanism of action of insecticides

Organochloride insecticde with DDT as an example, indicate that, the primary target site is the nervous system. DDT prolongs the inward sodium current and also suppress the increase in potassium permeability. A combination of these effects leads to the prolonged falling phase, increases the negative after-potential, and leads to repetitive activity (Hassall, 1982). The treated insects rapidly become hypersensitive to external stimuli and develop tremors of the body and appendages. After a period of violent motion they fall on their backs and the continuous leg movement eventually becomes more spasmodic, being succeed finally by paralysis. DDT can also affect the activity of ATPase (Matsumura, 1975).

Organophosphates and carbamates inhibits acetylcholinesterase activity. Acetylcholine is the transmitter at central nervous system synapses in insects. In order for the nervous system to operate properly it is necessary that, once the appropriate message has been passed, excess acetylcholine should be removed from the synapse, both to prevent repetitive firing of messages and to allow a succeeding message to be transmitted. This removal is effected by the enzyme acetylcholinesterase, which catalyses hydrolysis of the ester bond. The result of this inhibition is that acetylcholine accumulates in the synapses so that nerve function is impaired. This leads ultimately to the death of the insect (Devonshire *et al.,* 1992).

Neurotoxicity of pyrethroids has been attributed to their activity on the nervous system. Ion channels are the primary target sites for several classes of natural and synthetic pyrethroids (Bloomquist, 1996). The voltage-sensitive sodium channel is the major target site for DDT and pyrethroids. They cause the channels to close slowly after each action potential, leading to repetitive firing. Thus the membrane potential difference becomes more positive, so that the negative after-potential is increased (Bloomquist, 1996). Pyrethroid toxicologial significance on potassium channels when combined with the tendency of pyrethroids to keep sodium channel open, could enhance the ability of some pyrethroids to induce negative after-potential, repetitive firing, and nerve depolarisation (Miller and Salgado, 1985). Other channels inhibited by synthetic pyrethrin analogues include; Ca^{2+}-ATPase, Ca^{2+}+Mg^{2+}-ATPase and GABA-gated chloride ion channel receptors (Charlmers *et al.*, 1987).

2.4.3 Mechanism of insecticide resistance

Two major ways of resistance mechanisms have been identified. Firstly, is the reduced penetration of insecticide. This allows ample time for detoxifying enzymes to metabolize the chemical and therefore reduces effectivity. This kind of resistance was reported by Plapp and Hoyer in 1968 who found DDT and dieldrin resistance in *Musca domestica* was due to decreased penetration which leads to decreased absorption (WHO, 1994).

The second mechanism is the metabolic reistance. Metabolic pathways of the insect become modified in ways that detoxify the insecticide, or disallow metabolism of the applied compound into its toxic forms. The enzyme classes involved are the mixed function oxidases, esterases and glutathione S-transferases (WHO, 1994). Glutathione S-transferases catalyse the conjugation of glutathione with compounds having a reactive electrophilic centre, leading to the formulation of a water-soluble, less reactive product. Metabolism mediated by these enzymes has been implicated in DDT and organophosphate resistance. Monooxygenases complex involves a reductase, one or more cytochrome p450s and requires NADPH as cofactor (Devonshire *et al*., 1992). Cytochrome p450 enzymes are also involved in insect hormone and pheromone metabolism. Moreover, esterases are the most significant enzymes for insecticide detoxication in insects. Organophosphate, carbamate and pyrethroids contain carboxylester and phosphotriester bonds that are subject to attack by esterase enzymes.

Resistance to pyrethroids can be explained by two simple mechanisms. Firstly is the mutation caused by leucine substitution at position 1014 of the voltage-gated sodium channel. This leucine could either be substituted by phenylalanine or serine as seen in the West Africa and East Africa respectively. Leucine to phenylalanine substitution, involves the replacement of a non polar side chain for a larger aromatic side chain and this may impose steric constraints on the binding of the insecticide to the sodium channel. However serine substitution, which is a very small side chain, apparently also reduces the sensitivity of the sodium channel to insecticides. This suggests that mutations at this leucine may induce a conformational change in the hydrophobic segment 6 that affect pyrethroid binding. The substutited leucine is clearly not essential to the functioning of the insect sodium channel since non conservative substitutions at this site do not destroy its function. The replacement of the identical amino acid residue in many different insect species with knock down resistance suggests that the leucine residue is very important in the recognition of the pyrethroid insecticides (Ranson *et al*., 2000).

The second mechanism involves of cytochrome p450s. They are involved in the biosynthesis of several endogenous compounds and the detoxification of many xenobiotics (Nikou *et al*., 2003). Increased insecticide detoxification can result from elevated expression of one or more cytochrome p450s or from alterations in the primary structure of the protein. Certain cytochrome p450s have been associated with resistance of *An. gambiae* to pyrethroid resistance, for example is an adult specific CYP6 P450 gene: CYP6Z1 (Nikou *et al*., 2003), CYP6P3 and CYP6M2 (Djouaka *et al*., 2008).

2.4.4 Methods of detecting insecticide resistance

Detection of insecticide resistance is very important in monitoring of vector control. Certain methods are been implemented in vector control initiatives to monitor the effectiveness of the vector control strategy that is being enforced. The WHO bioassay involves the use of insecticide papers of known diagnostic concentrations, where the percetage mortality of the vectors will be established (Brown *et al*., 1986; WHO, 1992; Roberts and Andre, 1994). Biochemical and immunological bioassay are implemented in detection of insecticides resistance, and they focus on the enzymes associated with mechanims of resistance. They are achieved by conducting of

immunological tests or production of electrophoretic gels (Brown and Bradgon, 1987). DNA and RNA probes can also be implemented in detection of resistance. This molecular techniques are effecitive since one may be able to detect resistance even before it strikes. This involves the cloning of genes which encode for resistance proteins and tagging them with radiolabelled or nonradioactive labels. Thus, making it possible to detect the mutant allele that would confer resistance.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Selection for tolerance to cadmium and lead in *An. gambiae* ss

An. gambiae ss. mosquitoes were obtained from a colony kept in Arthropod Rearing and Containment Unit (ARCU) of International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. This colony was originally collected from Mbita field station (00.025'S, 34.013'E), Homa Bay County, Kenya in December, 2000, where *An. gambiae* ss. is abundant in nature. Cadmium and Lead heavy metal tolerant and control strains of the mosquito were separately generated and maintained through exposures to chronic concentrations of the metals in three independent replicates for each strain and treatment since 2006 as described by Mireji *et al*., (2010b). Briefly, Juvenile stages of *An. gambiae* ss. were selected for heavy metal tolerance in previously empirically determined, Maximum Acceptable Toxicant Concentrations (MATCs) of cadmium or Lead to the mosquito. Overall maintenance of the colony followed standard operating procedure for rearing *Anopheles* mosquitoes where female mosquitoes were blood-fed on anaesthetized mice and larvae on pulverized Tetramin fish food (Tetra GmbH, Melle, Germany) (Ford and Green, 1972). Approval for feeding mosquitoes on mice was obtained from the Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1), and the protocol reviewed by the KEMRI ACUC. Cadmium and lead used in this study were applied as cadmium chloride ($CdCl_2$) 99.99% pure (Fisher Scientific LLC, Fair Lawn, NJ, U.S.A.) and lead II nitrate [Pb $(NO_3)_2$] 99.5% pure analytical salts (Prolabo, Fontenay-sous-Bois, France).

3.2 Extraction of RNA and DNA from adult *An. gambiae* ss

Total RNA were separately isolated from pools of three independent replicates. Each pool consisted of five three-day-old adult mosquitoes (with male and female being handled separately) of cadmium or lead tolerant strain, or untreated control. The RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA) following manufacturer's protocol. Resultant RNA from each pool were separately suspended in 12µl of nuclease free water and stored at -80°C until required. Integrities of extracted RNA were validated by electrophoresis in 0.3% agarose (Sigma-Aldrich hemie, Gmbh, Steinheim, Germany) RNA denaturing gel in 1.4% sodium phosphate with 1 mg/ml ethidium bromide staining for visualization. The yields and qualities of RNA were determined spectroscopically (Sambrook *et al*., 1989). The genomic DNA was extracted from

similar pools of mosquitoes by ammonium acetate DNA extraction method with RNase (Bruford *et al*., 1998). The RNA and DNA extracted were subsequently used for cDNA synthesis and testing for heavy metal induced *kdr* mutation in the genomic DNA of the mosquito respectively**.**

3.3 Simultaneous species verification and molecular form characterization

Standard reactions were conducted as described by (Fanello *et al*. 2001). Briefly, 1µl of extracted DNA was amplified with 0.1u GenScript Taq DNA polymerase (GenScript Corporation, Piscataway, NJ) in a mixture of 2.0µl 10X GenScript buffer, 0.06mM dNTP and 0.5 picomoles of *An. gambiae* species identification primers (UN 5'-GTGTGCCCCTTCCTCGATGT-3' and GA 5' -CTGGTTTGGTCGGCACGTTT-3'). Reactions were carried out in PTC 100 programmable thermocycler (MJ Research, Gaithersburg, MD, USA). The cycling conditions involved 94°C for 3 minutes, 40 cycles of 94°C for 40 seconds, 55°C for 45 seconds, 72°C for 1 minute, and a final extension of 72°C for 10 minutes. After amplification of the *An. gambiae* specific band, 1 U of *Haemophilus haemolyticus* I (Hha I) restriction enzyme in 1Â SuRE/Cut Buffer L (Roche Diagnostic, Basel, Switzerland) was directly added to the PCR reactions and digestion carried out at 37ºC for a minimum of 3 h to enhance molecular identification of the *An. gambiae* detected earlier. Digested fragments were run through 2% agarose (Sigma-Aldrich hemie, Gmbh, Steinheim, Germany) gel in 1.4% sodium phosphate with 1 mg/ml ethidium bromide staining for visualization via UV-illumination. The gel photos were analyzed and documented using KODAK Gel Logic 200 Imaging System software (Raytest GmbH**,** Straubenhardt, Germany).

3.4 Quantification of cytochrome p450 genes

3.4.1 Reverse transcriptions

Two steps reverse transcriptions and DNase treatment to remove potential genomic DNA carry-over/contaminats were conducted using Revert Aid^{TM} H Minus first strand synthesis kit from (Fermentas, Lithuania) following manufacturer's instructions. The DNase treatment reaction consisted of 1µg RNA extract, 1µl 10X Reaction Buffer with $MgCl_2$, 1 u of RNase-free DNase I, and topped up with nuclease free water to make a total volume of 10µl. The reactions were incubated at 37°C for 30 minutes, followed by addition of 1µl 50mM EDTA and incubation for a further 10 minutes at 65°C. Resultant RNA from each strain were separately denatured by incubation at 65°C for 5 minutes in 12 µl reaction mix consisting of 1µg RNA, 1µl oligo $(dT)_{18}$

primer and 1µl nuclease free water. Subsequent cDNA synthesis were conducted by addition of 4µl 5X reaction buffer, 20 u RiboLock RNase inhibitor, 2µl 10 mM dNTP mix and 200 u Revert Aid H Minus M-MuLV reverse transcriptase in a total reaction volume of 20µl, followed by incubation at 45°C for 60 minutes, and the reverse trancriptase inactivated at 70°C for 5 minutes. The cDNA generated were stored at -20^{o}C.

3.4.2 Real-time qRT PCR of CYP6Z1, CYP6P3 and CYP6M2 CYP6 P450 enzymes

Expression profiles of CYP6Z1, CYP6P3 and CYP6M2 cytochrome p450 enzymes in the mosquitoes tolerant to lead or cadmium, and their respective control were separately assessed. Reaction mix consisting of 3µg cDNA template were separately amplified in three independent replicates with 7.5µl of Fast SYBR® Green master mix (Applied Biosystems, Carlsbad, CA) in presence of 0.5 picomoles specific primers for CYPZ1, CYP6M2, CYP6P3 and Ribosomal protein L19 (RPL19) internal control (Table 1). The reactions were carried out in Strategene MX3005P, real time qPCR machine (Aligent Technologies, California, USA) according to the manufacturer's instructions. The cycling conditions involved an initial step of 95°C for 30 seconds, 40 cycles of 95°C for three seconds, 60°C for 30 seconds, followed by one cycle of 95°C for 30 seconds, 60°C for one minute and 95°C for 30 seconds for all the genes. Crossing threshold values were recorded for all the sample reactions and subsequently used to quantify products of amplification using comparative Ct ($2^{-\Delta\Delta Ct}$) method (Margaret, 2010).

Table 1: Primers used for quantitative PCR studies

Cytochrome p450	GeneBankAccession number	Sequence (5'-3')	Product Size (bp)
CYP6M2	AGAP008212-RA	Fwd- TTCGTCGACTCTCCTCACCT	299
		Rev-GAAATGTACCGGGACTGGTG	
CYP6P3	AGAP002865-RA	Fwd-AGCTAATTAACGCGGTGCTG	121
		Rev-AAGTGTGGATTCGGAGCGTA	
CYP6Z1	AF487535	Fwd-TTACATTCACACTGCACGAG	146
		Rev-CTTCACGCACAAATCCAGAT	
RPL19	XM_001864829.1	Fwd-CCAACTCGCGACAAAACATTC	75
		Rev-ACCGGCTTCTTGATGATCAGA	

Fwd = forward primer, Rev = reverse primer, CYP6M2 = Cytochrome p450 family 6 gene M2, CYP6P3 = Cytochrome p450 family 6 gene P3, CYP6Z1 = Cytochrome p450 family 6 gene Z1, RPL19 = Ribosomal protein L19 Internal control

3.5 Detection of *kdr* mutation by PCR amplification sequencing

Standard reactions were conducted as described by Ranson *et al* (2000). Briefly, 1µg of template DNA from each replicate in the specific treatments were separately amplified with 0.2u GenScript *Taq* DNA polymerase (GenScript Corporation, Piscataway, NJ) in a mixture of 2.0µl 10X GenScript buffer, 0.6µl of 10mM dNTP and 0.5 picomoles of primer sets specific for detection of *kdr* pyrethroid resistance or susceptibility (Table 2). Reactions were carried out in PTC 100 programmable thermocycler (MJ Research, Gaithersburg, MD, USA). The cycling conditions involved 94°C for three minutes, 35 cycles of 94°C for 25 seconds, 55°C for 45 seconds, 72°C for one minute, and a final extension of 72°C for seven minutes. The amplified products were resolved by electrophoresis in 1% agarose (Sigma-Aldrich hemie, Gmbh, Steinheim, Germany) gel in 1.4% sodium phosphate with 1 mg/ml ethidium bromide staining for visualization via UV-illumination. Gel photographs were analyzed and documented using KODAK Gel Logic 200 imaging system (Raytest GmbH**,** Straubenhardt, Germany). The *kdr* amplicons were processed for sequencing using Quick Clean 5M Gel Extraction Kit (GenScript Corporation, Piscataway, NJ), following manufacturer's instructions, and were directly sequenced through Macrogen (Macrogen, Korea). Resultant chromatograms obtained were cleaned and edited, in order to create consensus sequences that were further aligned using BioEdit software (version 7.0.9) (Hall, 1999)

Table 2: Primer used for detection of pyrethroid *kdr* resistance or susceptibility mutation

Allele	Primer	Sequence 5'-3'	Product Size (bp)
Resistant	Agd1	ATAGATTCCCCGACCATG	195
	Agd5	TTTGCATTACTTACGACTG	
Susceptible	Agd2	AGACAAGGATGATGAACC	137
	Agd4	CTGTAGTGATAGGAAATTTA	

Agd1 = forward primer for resistance testing, Agd5 = reverse primer for resistance testing, Agd2= forward primer for susceptibility testing, Agd4 = reverse primer for susceptibility testing.

3.6 Bioassay for monitoring resistance

Bioassays for insecticide resistance were performed on cadmium or lead tolerant moquitoes, their respective reference controls, and on colony reared reference *An. gambiae* ss. (Kisumu strain) adults essentially following WHO standard protocols for insecticide susceptibility testing using filter papers impregnated with diagnostic doses of 0.75% permethrin (WHO, 2005). Every strain contained 8 replicates, each with 15 mosquitoes exposed to the permethrin impregnated papers in susceptibility testing kits for an hour, and then transferred into insecticide-free tubes for 24 hours. Mortality in each tube was subsequently recorded.

3.7 Data analysis

Crossing threshold values of expression profiles obtained from qPCR were used to calculate the relative expression values of the genes using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Statistical significant difference of gene expressions was determined by ANOVA using SPSS software. Data obtained from gene expression comparisons was presented in bar graphs and tables. Sequences of *kdr* were analyzed by BioEdit software program (version 7.0.9) to verify the presence of a leucine (TTA) or serine (TCA) substitution in case of susceptibility or resistance respectively. Multiple alignments created were used to verify presence or absence of this single base pair mutation within the region of interest. Data obtained from PCR amplification were presented as gel photo graphs. Vector response to insecticide was obtained by counting percentage mortality after 24 hours post exposure.

CHAPTER FOUR

4.1 Results

4.1.1 Expression profiles of metal responsive classes of CYP6 p450 genes

Relative expression profiles of CYP6M2, CYP6P3 and CYP6Z1 classes of CYP6 p450 genes in cadmium or lead tolerant *An. gambiae* ss, and their respective controls are summarized in Figure 1 and Table 3. Expressions of all the classes were significantly lower in females than males ($F_{(1, 46)}$=30.13 , p<0.0001) irrespective of treatment. Within gender, the reductions in expression were more pronounced in the male metal tolerant, relative to their respective control populations. There were general reductions in expression levels of all the genes in metal tolerant relative to control populations, where significantly lower expressions ($F_{(2, 46)}$= 7.06, p= 0.0021) of CYP6M2, CYP6P3 and CYP6Z1 genes were observed in the tolerant than in control male populations. There expression of all the genes in cadmium selected female populations was marginaly higher than in their respective controls, although the differences were not statistically significant (P > 0.05).

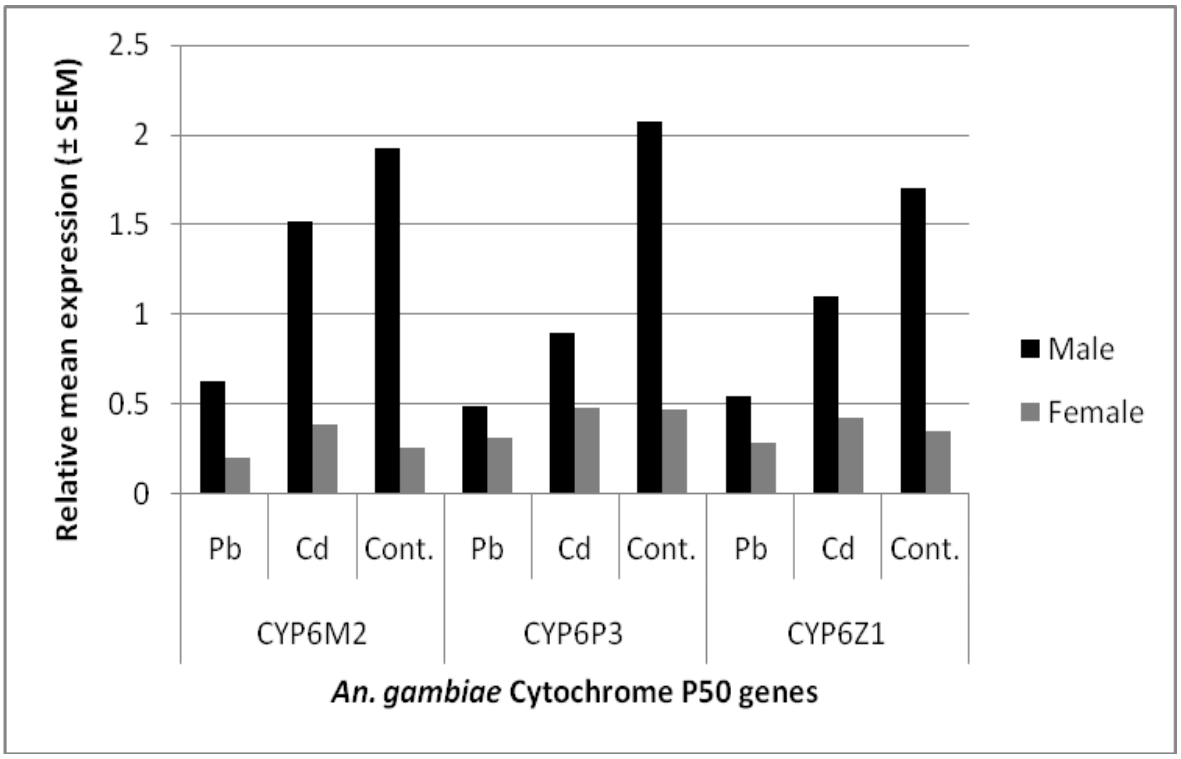

Figure 1: Sex specific influence of cadmium or lead tolerance on expressions of cytochrome p450 genes associated with pyrethroid resistance in *An. gambiae* mosquito vector of malaria

Table 3:. Relative mean (± SE) expression levels of various classes of cytochrome p450 genes in heavy metal tolerant adult *An. gambiae* ss mosquito in relation to controls

Gender	Treatment	CYP6M2	CYP6P3	CYP6Z1
Males	Cont.	1.927 ± 0.60^{a}	2.077 ± 0.964^{a}	1.704 ± 0.717^{a}
	Cd	1.516 ± 0.333^{ab}	0.898 ± 0.263^{ab}	1.104 ± 0.409^{ab}
	Pb	0.630 ± 0.193^{b}	0.490 ± 0.133^{b}	0.543 ± 0.118^{b}
Females	Cont.	0.253 ± 0.169^{bc}	0.470 ± 0.197^{bc}	0.346 ± 0.202^{bc}
	Cd	0.386 ± 0.152^{c}	0.482 ± 0.139^{c}	0.422 ± 0.093^{c}
	Pb	0.205 ± 0.049^{c}	0.307 ± 0.064^{c}	0.282 ± 0.077^{c}

Means with different superscripts within columns are significantly different ($p<0.05$). CYP6M2, CYP6P3 are CYP6Z1are classes M2, P3 and Z1 respectively in family 6 of cytochrome p450 genes. Cont., Cd and Pb represent control, cadmium and lead tolerant populations of *An. gambiae* ss.

4.1.2 Molecular forms and *kdr* genotypes in cadmium and lead tolerant *An. gambiae*

Molecular characteristics of cadmium or Lead tolerant *An. gambiae* ss are summarised in Figure 2. All the mosquiotoes were of M and S form irrespective of gender, and were susceptible to pyrethroid insecticide (Panel A). The multiple alighments of sequences from S6 transmembrane region of the voltage sensitive sodium channel sequence indicate presence of codon (TTA) specific for *kdr* suceptibility (Panel B).

4.1.3 Phenotypic behaviour of *An. gambiae* on exposure to insecticide papers

Mortality among all cadmium and lead tolerant mosquitoes, and their respective controls exposed to the permethrin were similar, within and between gender and treatments ($p > 0.05$). All the mosquito vectors died within the first 2 hours, during the 24 hours period of monitoring. However, cadmium tolerant female populations was unique where 2 mosquitoes managed to survive more than 6 hours after exposure to the insecticide papers. Mortality among cadmium tolerant female population was 98.5%.

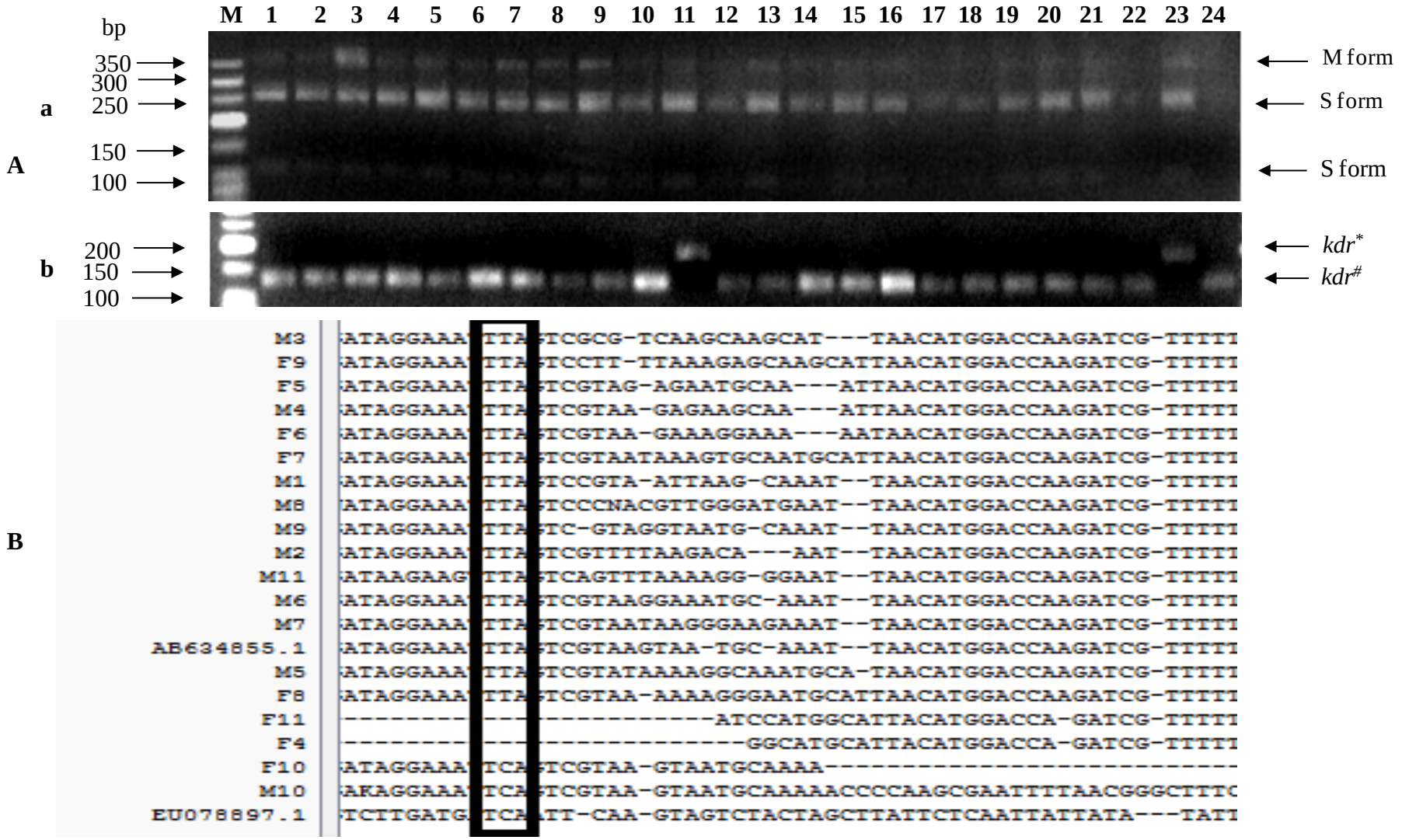

Figure 2: Molecular forms, knockdown resistance alleles (*kdr*) and associated mutations in cadmium or lead tolerant *An. gambiae s.s*

Panel Aa: Restriction digests using Hha1 enzyme for molecular characterization of DNA from male (1-8) and female (12 -20) *An. gambiae* tolerant to cadmium (1-4, 13-16)and lead (5-8,17-20) heavy metals, and controls not selected for metal tolerance (9 and 10, 21 and 22). Panel Ab: PCR amplification profiles of DNA with *kdr* diagnostic specific primers. Samples in lanes 11 and 23 represent pyrethroid resistant (kdr*) positive control while those in lanes 12 amd 24 represent pyrethroid susceptible (kdr$^{\#}$) positive controls. Panel B. Mutiple sequence local alignment of S6 transmembrane region of the voltage sensitive sodium channel, incorporating *kdr* of the *An. gambiae* ss strains. TTA or TCA of position 421 is indicative of pyrethroid susceptibility and resistance genoptypes respectively. AB634855.1 and EU078897.1 are published sequences for the S6 transmembrane region of the mosquito susceptible or resistant to the pyrethroids, respectively.

4.2 Discussion

CYP6M2, CYP6P3 and CYP6Z1 classes of CYP6 p450 genes were significatly ($p<0.0001$) down regulated by cadmium and lead tolerance in *An. gambiae* ss in a sex-specific manner, with the effect more pronouced among males within gender. Similar reductions in CYP6 P450 response to heavy metal tolerance/challenges have been observed in rats and fish (Gordon and Skett 2001; Henczova *et al*., 2003). These depressed expressions may be attributted to role of the metals in induction of heme oxygenase activity (cadmium) and inhbition of gamma aminolevilinic acid dehydratase enzyme (lead) which are involved in heme degradation and synthesis respectively (Gordon and Skett, 2001; Henczova *et al*., 2006; Henczova *et al*., 2008). These event effectively reduce heme concentration, a major component of CYP6 P450, and hence observed concomitant reduction in expression of the genes. Cadmium binding protein unique to females (Gordon and Skett, 2001) may be responsible for the differential responses of male and female mosquitoes to cadmium. The protein may be chelating the metal within the mosquito, thereby reducing bioavilability of the metals leading to induction of the CYP6 P450 due to reduced heme oxygenase activity. The metal tolerance did not induce *kdr* resistance mutation or permethrin resistance in the mosquito populations. This phenomanon can be attrubutted to relatively short selection duration insufficient to induce mutagentic changes in the mosquitoes. Alternatively, any mutagenic changes occationed by the metal tolerance did not affect genes associated with permethrin resistance, including the SNP related to the *kdr*. Phenotypic behaviour of the mosquito on exposure to permethrin papers confirm the inability of heavy metals to induce pyrethroid resistance.

Different patterns of expressions of various selected metals in males and females observed suggests existence of different biological processes operating in each metal. The significant decrease in effect of gene expression levels within various treatments in males than in females could be due to important function of the CY6 P450 genes in hormone metabolism or mating behavior (Nikou *et al*., 2003). This implies that CYP6M2, CYP6P3 and CYP6Z1 cytochrome p450 expression profiles in *An. gambiae* males can be employed as molecular markers for detection of heavy metal pollution, since the effect of heavy metal is detrimentous to animal and plant life. In addition, the heavy metal tolerant males have a significantly lower expression of these metabolising genes compared to their controls, whereby in heavy metal tolerant females, the gene expression are not significantly lower compared to their controls. This implies that the males are more susceptible to pyrethroid insecticide as compared to

females, thus, in a proliferation site polluted by heavy metals, it is expected that more female than male populations after administration of pyrethroid insecticide. This is an alarm for consern, since the female is our target for disease control and thus proper heavy metal disposal should be implimented to avoid the dominance of the female population. Moreover, the ability of heavy metal tolerant *An. gambiae* to have an effect on CYP6M2, CYP6P3 and CYP6Z1 classes of cytochrome p450 belonging to a family responsible for general insecticide resistance, gives a room for debate on how the heavy metals would affect the previously faced out insecticide groups or even the future insecticides. Thus, in case of any new insecticide developments, it is recommendable for quantitative expression of various classes of this CY6 P450 family to be conducted on heavy metal tolerant *An. gambiae* mosquito in order to understand how the insecticide would affect insects within the heavy metal polluted environments. The susceptibility of the heavy metal tolerant *An. gambiae* ss, discredits the general campaign against use of bed nets and thus encourages the control measure against malaria to be implemented atleast in heavy metal polluted environments.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

1. CYP6M2, CYP6P3 and CYP6Z1 classes of CYP6 p450 genes were significatly down regulated by cadmium or lead tolerance in a sex-specific manner, with the effect more pronouced among males within gender, probably due to their different mating behaviors and hormone metabolism.
2. Cadmium or lead tolerance did not induce *kdr* resistance mutation or permethrin resistance phenotype in the mosquito populations. Implying that despite the ability of heavy metal to induce mutations, it was not able to cause the TTA to TCA single nucloetide polymorhism substitution that infers pyrethroid resistivity.
3. The cadmium or lead heavy metal tolerant *An. gambiae* ss are more susceptible to pyrethroid insecticide as compared to their female counterparts. Thus suggesting efficacy of the use of pyrethroid insecticide as a vector control in regions polluted by heavy metals, where more males will be killed as compared to females.

5.2 Recommendation

1. The National Environment Management Authority (NEMA) body is advised to ensure that the contamination of the water collection sites by human activities in the urban environment should be stop / minimized, because for sure the vector has managed to adapt and survive the polluted environment, a trait which is influencing a subsequent response to pyrethroid insecticide resistance. This need to be maintained until a thorough investigation is conducted with certainty, to investigate the exact contaminants in this water collection sites that is actually responsible for the cross pyrethroid resistance observed in nature.
2. Functional genomics studies involving silencing of known and novel metal responsive genes in CYP6 p450 metabolism pathway will shed light on the underlying mechanism regulating expression of the genes in lead tolerant mosquitoes. This is very importanat since, it will contribute to the understanding of how to improve the pyrethroid insecticide, which is a very improtant step in ensuring successful vector eradication and disease control as compared to introduction of a new insecticide which will also be bound to resistance development.
3. Detailed HPLC technique on soil and water in this habitats to understand the exact contaminant that yields this cross resistance to pyrethroid insecticides, as portrayed by

other research scientists in Western Africa, who showed that urban pollution results to cross resistance to pyrethroid insecticide by introduction of the *kdr* mutation or over expression of the cytochrome P450 genes.

4. Further exploration as to why the genetic expression was in a sex specific manner, yet this was not shown in the phenotypic response bioassay. This has to be investigated so as to have a clear understanding of the mechanism of the observed pyrethroid susceptibility induction: down regulation of the pyrethroid metabolizing genes.
5. A further comparison with the wild species from the field (mbita station) need to be conducted so as to investigate whether the colonized laboratories used in this experiment, gives a glimpse of what is actually there in the ground.
6. Investigation as to how the influence of heavy metal adaptation on the subsequent affectivity of the vector to transmit *Plasmodium falciparum*, will be also very important especially when it comes to malaria disease control.

CHAPTER SIX
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APPENDICES

Ammonium Acetate Precipitation Protocol based on Bruford *et al*. (1998)

1) In a 1.5ml flip-top clean STERILE (ie Autoclaved) eppendorf tube place 250ul of Digsol buffer and 10ul of 10mg/ml Proteinase K (stored at -20°C). Keep on ice.

Digsol buffer (stock should be fresh and Sterile (AUTOCLAVED !) stored at room temp.

RECIPE

DIGSOL

To make 1L:

Final concentration required	Volume/amount required of stock reagents
20mM EDTA	40ml of 0.5M EDTA (pH 8.0)
120mM NaCl	6.85g solid Sodium Chloride (NaCl)
50mM Tris.HCl	50ml of 1M, Tris.HCl (pH 8.0)
Water	810 ml of double distilled autoclaved water

Ensure all constituents are dissolved.

Autoclave at 15psi for 20 minutes.

Add 50ml of 20% SDS (Sodium lauryl sulphate).

pH to 8.0 with concentrated HCl if needed.

Final volume = 1 litre.

2) Centrifuge blood sample at 13,000rpm for about 1 min.
3) Remove a blob of blood (or animal tissue) from the ethanol in which it is stored with a STERILE (autoclaved) toothpick and blot onto clean tissue.

When the blood on the toothpick is blotted dry, place it the eppendorf containing the Digsol buffer and jiggle the toothpick to dislodge the blood. When the blood has fallen into the Digsol buffer, remove toothpick and discard by placing in bleach.

4) Vortex for 30 seconds, wrap the rack in tissue and cling-film and place in rotating oven at 55 (3hrs) or 37 degrees centigrade overnight.
5) Once digested (when digested the solution becomes a straw colour) add 300ul 4M ammonium acetate to each eppendorf.
6) Vortex for 30 seconds several times over a period of at least 15 minutes at room temp.
7) Centrifuge for 15 minutes at 15,000rpm (15 minutes and 13,000-15, 000 rpm are needed to ensure the DNA is spun to the bottom of the eppendorf – this is based on the size of DNA – Ensure your centrifuge it set to 13,000rpm or is manufacturer to reach this speed.

8) Transfer supernatant (clear liquid) into clean sterile (autoclaved) labelled 1.5ml eppendorf by using a P1000 pipette (discard the original eppendorf which now contains only the gunky protein stuff).
9) Add 1ml 100% ethanol to your supernatant using a P1000 pipette.
10) Vortex 2 seconds and then invert eppendorf gently 10-20 times to precipitate DNA – DNA may be seen as white spiral threads if it is concentrated or a cloudy white precipitant but don't worry if no change is observed.
11) Centrifuge for 15 minutes at 15,000rpm
12) Pour and the pipette off ethanol taking care not to lose DNA pellet – the pellet is your DNA ! – Ensure all ethanol is removed using a P200 pipette (otherwise future PCRs and restriction digests will be inhibited.)
13) Add 900ul 70% cold ethanol (stored at –20°C) and invert gently 10-20 times to rinse pellet (removes the salt which also inhibits future reactions).
14) Pour and the pipette off ethanol taking care not to lose DNA pellet – the pellet is your DNA ! – Ensure all ethanol is removed using a P200 pipette (otherwise future PCRs and restriction digests will be inhibited.)
15) If the pellet dislodges from the bottom of the tube centrifuge for 5 minutes at 15,000rpm to fix it back to the bottom of eppendorf.
16) Perform a second -70°C rinse. Add 900ul 70% cold ethanol (stored at –20°C) and invert gently 10-20 times to rinse pellet (removes the salt which also inhibits future reactions).
17) Pour and the pipette off ethanol taking care not to lose DNA pellet – the pellet is your DNA ! – Ensure all ethanol is removed using a P200 pipette (otherwise future PCRs and restriction digests will be inhibited.)
18) If the pellet dislodges from the bottom of the tube centrifuge for 5 minutes at 15,000rpm to fix it back to the bottom of eppendorf.
19) Pour off ethanol and stand eppendorfs open underneath a desk lamp for 30 minutes to dry the pellet. Pellet appears as a white flake of skin when dry as opposed to a translucent piece of gel when wet. It must be dry before proceeding.
20) Once fully dry add 100ul -300ul T10 E0.1. or sterile (double distilled) autoclaved water to resuspend the pellet.

Add 100ulif a small amount of blood was used and a small pellet observed.

Add 300ul is a big amount of blood was used and a large pellet observed.

NOTE.

It is essential the TE is freshly made and has been AUTOCLAVED so is Sterile !

TE (Stock should be fresh and Sterile (AUTOCLAVED !). Stored at room temp)

RECIPE

Low TE ($TE_{10}E_{0.1}$) (pH 7.5-8.0) for making up 400ml

Concentration required	Volume of stock reagents required
10mM Tris.HCl	4ml of 1M Tris.Hcl (pH 8.0)
0.1mM EDTA	80ul (NB MICROLITRES!) of 0.5M EDTA (pH 8.0)
Water	396ml of double distilled autoclaved water

Final Volume = 400ml

No necessary to pH

Autoclave 15psi for 20 minutes

21) Briefly vortex (2 second blasts) and flick sample 5-10 times to dislodge pellet
22) Place eppendorfs in waterbath for 30 minutes (37 or 65°C) to dissolve pellet (flicking every 10 minutes)
23) Ensure pellet is suspended using a P1000 pipette to mix the DNA and TE – it is essential the DNA is fully dissolved. If the DNA solution is still glupey – it is highly concentrated – This can be checked on a 0.8% agarose check and 100ul more sterile TE added.
24) Store at –20C degrees.

The DNA is stored at –20°C to prevent DNAases from digesting and degrading the DNA. (DNAases are present on your skin and any non-sterile solutions and would if present digest the entire DNA in your eppendorf – leading you to loosing the whole extracted DNA sample). The DNA can be kept at 4 degrees during the day for a maximum of 1 day. DNAases can be active at 4°C so storing for longer than one day not recommended. Best to return to -20°C freezer each time after use.

Trizol Invitrogen method for RNA extraction

1) Add TRIzol to the sample and homogenize.

Homogenize sample using a glass Teflon or power homogenizer (Polytron or Tekmar's Tissumizer) in a 1.5 microcentrifuge tube. Tissue culture cells do not have to be homogenized, but just mixed extensively with TRIzol. Sonication will work to lyse cells in TRIzol, but should only be used if the researcher does not plan to isolate DNA from the sample. Cells grown in monolayers can be lysed directly in the culture dish.

2) Add chloroform to the homogenate. Use 200ul of chloroform for every 1ml of TRIzol used. Invert and mix well.

Use straight chloroform; no isoamyl alcohol is needed. Chloroform with 50 ppm amylene can be used. Chloroform solublizes lipids in the sample.

3) Centrifuge to get phase separation.

Using lower centrifugation speeds: Centrifugation speeds as low as 5000 -6000 x g have been used at this step, however, the centrifugation time should be doubled to 30 min. to get similar yields. Multiple phases observed upon addition of chloroform: If you observe multiple phases after adding chloroform and mixing, these are most likely due to inadequate mixing of the chloroform with the reagent. Chloroform is much more dense than TRIzol and therefore will immediately sediment to the bottom of the tube. Simply vortexing the solution will not properly mix chloroform with the TRIzol solution and may give a partial phase separation. Whenever clear water-like solution on the bottom of the tube is observed, this may be chloroform. Simply remix the samples by shaking the contents vigorously as stated in the protocol and centrifuge the samples at 12000 g for 15 min. at 4ºC. The 4°C spins are essential for phase separation. Room temperature spins may result in variable phase separation thus resulting in variable RNA yields.

4) RNA is in the upper aqueous phase. Remove upper aqueous phase and precipitate to get the total RNA.

5) Use 0.5 ml isopropyl alcohol for every 1ml of TRIzol used.

If isopropanol is inadvertently added at this step instead of chloroform, add more isopropanol to precipitate everything, then resuspend the pellet in TRIzol and use the protocol as specified. RNA yields will be compromised, but it may be possible to obtain a product in RT-PCR.

Protocol for adding more isopropanol if isopropanol was used instead of chloroform:

a) Add more isopropanol so that the total volume of isopropanol equals the volume of TRIzol used. Spin at 7500 x
b) g for 10 min. at 4°C.
c) Pour off supernatant; allow relatively compacted pellet to air dry Estimate the size of the pellet in µl; add at least
d) 15-20 volumes of TRIzol (e.g., for a 100 µl pellet, add at least 1.5 ml TRIzol).
e) Break the pellet up well (may have to use a hand-held homogenizer). Store the solution for 10-15 min. at room
f) temperature; every 5 min. or so, shake it by hand to make certain it is well dispersed.
g) Proceed with the TRIzol protocol as written (i.e., add chloroform). Results will not be optimal, but it may be
h) possible to get a product in RT-PCR.

6) Incubate and centrifuge.

7) Wash RNA pellet with 75% ethanol l for every 1 ml of TRIzol used. Mix sample by flicking and inverting the tube or vortexing and centrifuge at 7500 x g for 5 min. at 4°C.

8) Redissolve the RNA pellet.

9) Long-term storage of RNA: For long-term storage, it is recommended to resuspend RNA in stabilized formamide and storing at -70°C. To remove the formamide, add 4 volumes ethanol, and if less than 20 µg RNA also add NaCl to 0.2 M. Precipitate the RNA.

10) For spectrophotometric readings or gel electrophoresis, the RNA may remain in formamide. For RT-PCR, the RNA should be diluted to a final concentration of 5%.

When Isolating RNA from small quantities of tissue the following protocol may be used:

a) . Add 800 µl TRIzol to the sample. Homogenize cells by pipetting repeatedly. Add 200 µg glycogen (Cat. No. 10814-010) directly to the TRIzol reagent. If processing tissue, pulverize in liquid nitrogen first and then add 800 µl TRIzol containing 200 µg glycogen (final concentration 250 µg/ml) followed by vigorous vortexing or power homogenization.
b) Place at room temperature, cap vial, and vortex at high speed for 10 sec. Make sure the TRIzol reagent wets the side of the vial in order to solubilize any sample that may be remaining on the walls.
c) Shear the genomic DNA in the sample by passing twice through a 26-gauge needle connected to a 1 ml syringe. Using the syringe, transfer the sample to a sterile 1.5 ml microcentrifuge tube.

d) Add 160 µl of chloroform (or 49:1 chlorform:isoamyl alcohol) to each sample and vortex up to 30 s. Spin at maximum speed in the microcentrifuge 5 minutes to separate the phases.
e) Transfer the upper aqueous phase to a fresh tube and add 400 µl ice-cold isopropanol.
f) Allow the samples to precipitate at -20°C 1 hour - overnight. Pellet the RNA by centrifugation at maximum speed in the microfuge 15 min at room temperature.
g) Decant the supernatant.
h) Wash the pellet in 200 µl of 70% ethanol and spin again 10 min at maximum speed.
i) Decant the supernatant, removing as much as possible without disturbing the pellet. Dry RNA pellet.
j) Resolubilize the pellet in 30 - 50 µl RNAse-free deionized water. NOTE: If tissue is high in RNAses (e.g., adrenal gland, pancreas,) resuspend in 100% deionized formamide. Be sure to vortex or pipette up and down the sample to ascertain that pellet is resolubilized fully.
k) Store at -70°C

Table 4: Yield and quality of extracted RNA of adult *An. gambiae* ss mosquito determined by spectroscopy

Sample Name	Yield (µg/ml)	Quality (260nm/280nm)
M1	659.28	2.98
M2	615.53	2.42
M3	583.74	2.12
M4	577.98	2.22
M5	579.30	2.46
M6	566.21	1.88
M7	556.35	2.25
M8	524.37	2.76
M9	559.38	2.00
F1	551.66	1.67
F2	563.38	1.96
F3	538.62	1.97
F4	548.54	2.05
F5	560.21	1.89
F6	559.23	2.20
F7	549.41	1.40
F8	559.86	2.06
F9	548.0	2.26

Samples whose name proceed with the letter M and F are male and female mosquitoes respectively. Samples whose name bear the numbers: 1-3 (M1-M3 and F1-F3), are independent replicates exposed to lead treatment; 4-6 (M4-M6 and F4-F6), are independent replicates exposed to cadmium treatment; 7-9 (M7-M9 and F7-F9), are independent replicates of the untreated control

Table 5: Analysis of variance results of the effect of the gene, gender, treatment and gender-treatment interaction factors on the linear model

Factor	df	SS	MS	F -=value	P-value
Gene	2	0.07	0.03	0.10	0.9032
Gender	1	9.97	9.97	30.13	<.00001
Treatment	2	4.67	2.34	7.06	0.0021
Gender*Treatment	2	3.65	1.82	5.51	0.0072
Residuals	46	15.23	0.33		

df=degrees of freedom; SS=sum of squares; MS= mean of squares; *= interaction term.

Table 6: Parameter estimates and their associated p-values, showing pair-wise mean comparison within gene, gender and treatment factors

Covariate	Estimate	Std. Error	t-value	p-value
Intercept	1.94	0.22	8.77	<.0001
Gene				
CYP6M2	_			
CYP6P3	-0.03	0.19	-0.168	0.8676
CYP6Z1	-0.09	0.19	-0.447	0.6568
Gender				
Male	_			
Female	-1.55	0.27	-5.702	<.0001
Treatment				
Control	_			
Lead	-1.35	0.27	-4.971	<.0001
Cadmium	-0.73	0.27	-2.692	0.0099

Table 7: Detailed pair-wise mean comparison of gender-treatment interaction within the various treatments

	Male			Female		
	Control	Lead	Cadmium	Control	Lead	Cadmium
Control	1	-	-	1	-	-
Lead	-1.348 (p<.0001)	1	-	-1.091 (p=0.999)	1	-
Cadmium	-0.730 (p=0.0096)	0.618 (p=0.224)	1	0.074 (p=1.000)	0.165 (p=0.990)	1