DIFFERENTIAL TRANSCRIPT AND PROTEIN EXPRESSION PROFILES IN *ANOPHELES GAMBIAE SENSU STRICTO* MOSQUITO LARVAE EXPOSED TO CADMIUM HEAVY METAL

NGAMBI CATHERINE NYAWIRA

A Thesis Submitted to the Graduate School in Partial fulfilment of the Requirements for the Doctor of Philosophy Degree in Biochemistry of Egerton University

EGERTON UNIVERSITY

NOVEMBER, 2021

DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented in this university or any other for the award of a degree.

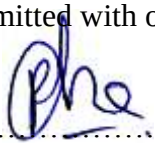
Signature… ……Date…22nd October 2021……

Catherine Nyawira Ngambi

SD14/0323/11

Recommendation

This thesis has been submitted with our approval as university supervisors.

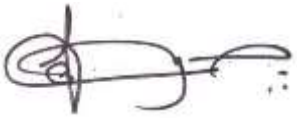


Signature……Date…22nd October 2021……

Dr. Paul O. Mireji,

Kenya Agricultural and Livestock Research Organization (KALRO), Muguga, Kenya.

P.O Box 362-00902, Kikuyu, Kenya.



Signature…… ……Date……22nd October 2021……

Prof. Francis N. Wachira,

South Eastern Kenya University, Kitui, Kenya.

P.O Box 170-90200, Kitui, Kenya.

Signature…… ….Date……22nd October 2021……

Dr. Daniel K. Masiga

International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya

P.O Box 30772-00100, Nairobi, Kenya.

DEDICATION

I dedicate this work to my husband, Mr. Stephen Muturi and our children, Victor, Amani, Nathan and Esther who have been there for me and have been an inspiration to me throughout the study period. I also dedicate this work to the entire Ngambi's family especially my dad, Mr. George Ngambi who was sceptical about me pursuing a PhD. Finally, I dedicate this work to my cousins Ms. Mercy Muturi and Ms. Elizabeth Muturi for their love, concern and encouragement throughout this journey.

ACKNOWLEDGEMENTS

I thank the Almighty God for giving me the opportunity to study and granting me divine health and grace to complete my PhD studies successfully. I sincerely extend gratitude to my supervisors; Dr. Paul Mireji, Prof. Francis Wachira and Dr. Daniel Masiga for their guidance, support and encouragement. I thank Dr. Paul Mireji for assisting me secure funding from the Deutscher Akademischer Austausch Dienst (DAAD) (German Academic Exchange Service) fellowship and together with Dr. Ramadhan Mwakubambanya for their assistance in obtaining additional research grant support from the Division of research and extension of Egerton University, Njoro, Kenya. I also appreciate Dr. Daniel Masiga for assisting me secure funding by the World Federation of Scientists (WFS). I thank the entire Molecular Biology and Biotechnology Department at the International Centre of Insect Physiology and Ecology (ICIPE) under the leadership of Dr. Daniel Masiga for providing laboratory space and allowing me to use laboratory equipments and consumables for the entire period of my research work. I thank Dr. Martin Rono at the Kenya Medical Research Institute (KEMRI), Kilifi County, Kenya, for his mentorship in the proteomics work. My sincere gratitude also goes to Mr. Richard Ochieng' for his assistance in the rearing of mosquitoes in the insectary unit at ICIPE.

I express my gratitude to DAAD for granting me a scholarship that catered for the tuition fees and research component of this endevour thereby enabling this work to be completed. I thank Egerton University, Njoro, Kenya through the office of the Registrar (Human Capital and Administration) and graduate school for granting me tuition waiver and giving me an opportunity to pursue a PhD degree in Biochemistry. I am grateful to my colleagues in the Molecular Biology and Biochemistry Department of the Egerton University for their moral support, advice and encouragement during this period. I am thankful to my prayer partner Jecinta Wanjiku Kibira for her prayer support in this endeavour. I am also grateful to my sisters Susan and Eunice for their moral support, concern and accommodation during my research work.

I am grateful to my spiritual father, Apostle John Kimani William and his wife Rev. Naomi Kimani for their unending prayers to release destinies and careers that has seen me through this far. To all, mentioned and not mentioned, who participated in one way or the other in this journey, I say thank you and may God richly bless you.

ABSTRACT

Anopheles gambiae sensu stricto mosquito larvae are known to thrive in non-polluted environments. However, the larvae have adapted to heavy metal polluted habitats at a significant biological cost. The molecular processes mediating the adaptation are poorly understood. This study assessed transcriptional and proteomic responses in *An. gambiae s. s* larvae following exposures to cadmium heavy metal as proxy assessment of adaptation of the mosquito to heavy metals. Three independent replicates of third instar larvae were exposed to cadmium or not (controls). Their RNAs were separately extracted and complementary DNA (cDNA) were synthesised from the RNA. Differentially expressed transcripts among cDNA transcripts were identified using the Annealing Control Primer (ACP) technology. Similarly, protein was concurrently extracted in triplicates from third instar larvae (n=50), separated using SDS gel and in-gel protein digestion using trypsin. The protein fragments were then analysed and profiled using Mass spectrometry (MS) and a suite of bioinformatics software. Transcriptomic analysis identified fourteen differentially expressed genes, of which eleven were up-regulated by the cadmium exposure while three were down-regulated. The up-regulated genes were clustered into biological functions that encompassed metabolism, transport and protein synthesis. The down-regulated transcripts included Protein G12, adenylate cyclase and endoplasmic reticulum metallopeptidase. Proteomics analysis revealed the down-regulation of immunity, protein synthesis and degradation and proton transport proteins by cadmium exposure in the mosquito larvae. There was an up-regulation of proteins with catalytic activity in the cadmium exposed larvae. There was also an up-regulation of signalling molecules of the small GTPase family. Other proteins induced included, transcription factor, ribosomal proteins and those in the protein degradation pathway involving ubiquitination. These findings provide information of cadmium responsive transcripts and proteins that are useful in designing more effective vector control methods that match changes in vector dynamics.

TABLE OF CONTENTS

DECLARATION AND RECOMMENDATION ..... **ii**

COPYRIGHT ..... **iii**

DEDICATION ..... **iv**

ACKNOWLEDGEMENTS ..... **v**

ABSTRACT ..... **vi**

LIST OF FIGURES ..... **xi**

LIST OF TABLES ..... **xii**

LIST OF ABBREVIATIONS AND ACRONYMS ..... **xiii**

CHAPTER ONE **1**

INTRODUCTION **1**

1.1 Background Information 1

1.2 Statement of the Problem 5

1.3 Objectives 6

1.3.1 General Objectives 6

1.3.2 Specific Objectives 6

1.4 Hypotheses 6

1.5 Justifications of the Study 7

1.6 Scope and Limitations 8

CHAPTER TWO **9**

LITERATURE REVIEW **9**

2.1 *Anopheles gambiae* species complex 9

2.2 The life cycle of the Mosquito vector 10

2.3 Malaria parasites 12

2.4 The life cycle of malaria parasites 13

2.5 Clinical manifestations of *P. falciparum* infection 15

2.6 Burden of Malaria Disease 15

2.7 Mosquito Larval Habitats 16

2.8 Malaria Transmission 18
2.9 Effects of human activities on malaria vector population 18
2.10 Methods used to control mosquito vector of malaria 19
2.10.1 Insecticides 20
2.10.2 Repellents 22
2.10.3 Ethnobotanical plants with phytochemicals 23
2.11 Control of *Anopheles* Larvae in Malaria Control Initiative 24
2.11.1 Biological control 25
2.11.2 Chemical larvicides 26
2.12 Genetic Control of Mosquitoes 27
2.12.1 Sterile Insect Technique (SIT) 27
2.12.2 *Wolbachia* 28
2.12.3 Genetic Modification of mosquitoes through Gene Drive 29
2.13 Insecticide resistance 30
2.14 Environmental Pollution and Ecology of Malaria Vector 31
2.15 Heavy metal pollution in Kenya 33
2.16 Methods used in differential gene expression 34
2.16.1 Gene Fishing Technique 34
2.16.2 Subtractive hybridization 35
2.16.3 Bulk Segregant Analysis (BSA) 36
2.16.4 Microarrays 38
2.16.5 RNA Sequencing 39
2.17 Proteomics 41
2.17.1 History of proteomics 41
2.17.2 Separation of proteins and peptides 44
2.17.3 Chromatography-based separation methods 45
2.17.4 Visualization of proteins 50
2.17.5 Mass spectrometry ionization methods 52
2.17.6 Types of mass analysers 53
2.17.7 Mass spectrometry-based quantitative proteomics 56
2.17.8 Peptide Mass Fingerprinting (PMF) 59

CHAPTER THREE **60**
MATERIALS AND METHODS **60**
3.1 Establishment of cadmium tolerant and control untreated colony of *An. gambiae s. s* 60
3.2 RNA Extraction 61
3.2.1 RNA extraction using ZR Tissue and Insect Microprep kit 61
3.2.2 RNA extraction using the Trizol reagent 62
3.3 DNase I digestion 62
3.4 RNA Quantification 63
3.5 Gene Fishing™ Reverse Transcription 63
3.6 Annealing Control Primer-based Gene Fishing™ PCR 63
3.7 Gel Extraction 64
3.8 Cloning 64
3.9 Sequencing 65
3.10 Protein expression studies 65
3.10.1 Test insects 65
3.10.2 Protein extraction 66
3.10.3 Liquid chromatography and MS/MS of *An. gambiae s. s* larvae proteome 66
3.10.4 Differential protein abundance analysis 67

CHAPTER FOUR **68**
RESULTS **68**
4.1 Establishment and maintenance of cadmium tolerant and control colony 68
4.2 RNA Quantification 68
4.3 Differentially Expressed Gene transcripts in *An. gambiae s. s* 70
4.4 Protein Expression in *An. gambiae s.s* larvae 72
4.4.1 Functional enrichment and network analysis 79
4.4.2 Down regulated proteins in cadmium treated mosquito larvae 81
4.4.3 Proteins induced by cadmium exposure in *An. gambiae s. s.* larvae 81

CHAPTER FIVE 82
DISCUSSION 82
5.1 *An. gambiae s. s* colony establishment and maintenance 82
5.2 RNA Quantification 82
5.3 Differential transcript profiles in *An. gambiae s. s* 82
5.4 Differential protein profiles in *An. gambiae s. s* 84

CHAPTER SIX 89
CONCLUSIONS AND RECOMMMENDATIONS 89
6.1 Conclusions 89
6.2 Recommendations 89

REFERENCES 90
APPENDICES 143
Appendix A. Total proteins identified from *An. gambiae s. s* L3 larvae 143
Appendix B. Proteins down regulated in cadmium treated mosquitoes 177
Appendix C. Proteins induced after cadmium treatment 187
Appendix D: Publications 189
Appendix E: Research Permit 191

LIST OF FIGURES

Figure 1: *Anopheles gambiae*, malaria vector 10

Figure 2: The life cycle of a mosquito 13

Figure 3: The life cycle of *Plasmodium falciparum* 15

Figure 4: Differential banding patterns in cadmium treated and control population of mosquito larvae 72

Figure 5: Venn diagram representing mosquito L3 larvae proteins in both cadmium tolerant and control populations and those unique to each 74

Figure 6: Principal Component Analysis of the replicate samples for cadmium tolerant versus the control group 75

Figure 7: Heat map illustrating proteins differentially expressed in cadmium exposed larvae versus the control group 76

Figure 8: Functional enrichment of differentially regulated proteins 80

LIST OF TABLES

Table 1: RNA quantification using the micro-spectrophotometer of cadmium and control samples extracted either using Trizol reagent or ZR Insect and Tissue RNA Kit 69

Table 2: RNA quantification of control samples (CT) and cadmium tolerant samples (CD) 70

Table 3: Gene transcripts obtained through BLAST searches from VectorBase..... 73

Table 4: Differentially expressed *An. gambiae s. s* larval proteins after exposure to cadmium heavy metal 77

Table 5: Biological pathways down-regulated in *An. gambiae s. s* larvae after exposure to cadmium heavy metal 81

LIST OF ABBREVIATIONS AND ACRONYMS

ACP	Annealing Control Primer
ACT	Artemisinin Combined Therapy
ACUC	Animal Care and Use Committee
AFLP	Amplified Fragment Length Polymorphism
ARQU	Animal Rearing and Quarantine Unit
BLAST	Basic Local Alignment Search Tool
BSA	Bulk Segregant Analysis
Bti	*Bacillus thuringiensis israelensis*
cAMP	Cyclic Adenosine Monophosphate
Cas9	CRISPR-associated protein 9
CDC	Centre for Disease Control
cDNA	Complementary DNA
CI	Cytoplasmic Incompatibility
CID	Collision-Induced Dissociation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSA	Chondroitin Sulphate A
DAAD	Deutscher Akademischer Austausch Dienst
DC	Direct Current
DDT	Dichlorodiphenyltrichloroethane
DEG	Differentially Expressed Gene
DML	dimethyl labelling
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECD	Electron Capture Dissociation
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological inoculation rate
ESI	Electron Spray Ionization
ETD	Electron Transfer Dissociation
FA	Formic acid

FDR	False Discovery Rate
FTICR	Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
HCD	High-energy Collisional Dissociation
HPLC	High Performance Liquid Chromatography
HSP	Heat-shock proteins
IAA	Iodoacetamide
ICAT	Isotope-Coded Affinity Tag
ICIPE	International Centre of Insect Physiology and Ecology
ICPL	Isotope Coded Protein Labelling
IEC	Ion Exchange Chromatography
IEF	Isoelectric Focusing
ILRI	International Livestock Research Institute
IMAC	Immobilised Metal-Ion Affinity Chromatography
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
iTRAQ	Isobaric Tags for Relative and Absolute Quantification
KEMRI	Kenya Medical Research Institute
KNBS	Kenya National Bureau of Statistics
L3	Third instar larvae
LC	Liquid Chromatography
LFQ	Label Free Quantification
LLIN	Long-Lasting Insecticidal Nets
MALDI	Matrix Assisted Laser Desorption Ionization
MD-LC	Multi-Dimensional Liquid Chromatography
MOP	Malaria Operational Plan
mRNA	Messenger RNA
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NCBI	National Centre for Biotechnology Information
ng	Nano gram
NGS	Next Generation Sequencing

NMCP	National Malaria Control Program
PAGE	Polyacrylamide Gel Electrophoresis
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pI	Isoelectric Point
PMF	Peptide Mass Fingerprinting
PMI	President's Malaria Initiative
PRR	Pattern Recognition Receptor
qPCR	Quantitative Polymerase Chain Reaction
Q-TOF	Quadrupole- Time of-Flight
RAPD	Randomly Amplified Polymorphic DNA
RBC	Red Blood Cell
RBM	Roll Back Malaria
RF	Radio Frequency
RFLP	Restriction Fragment Length Polymorphisms
RNA Seq	RNA Sequencing
RNA	Ribonucleic Acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
RP	Reversed Phase
RPM	Revolutions per Minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RUMA	Rapid Urban Malaria Appraisal
SCX	Strong Cation Exchanger
SDS	Sodium Dodecyl Sulphate
SEC	Size Exclusion Chromatography
SILAC	Stable Isotope Labelling with Amino acids in cell Culture
SIT	Sterile Insect Technique
SNP	Single Nucleotide Polymorphisms
TMT	Tandem Mass Tags
TOF	Time of Flight

UN	United Nations
UNPF	United Nations Population Fund
UV	Ultra Violet
WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Malaria is a global disease which is so devastating and it threatens almost half of the population globally. In sub-Saharan Africa, this disease causes high mortality and morbidity inspite of huge investments in its control (WHO, 2013). More than 200 million incidences of malaria were reported annually, with an estimated half a million people succumbing to the disease in 2012 (WHO, 2013). Ninety five countries in the year 2015 were having malaria transmissions whereby approximately 3.2 billion people were facing the risk of this infection and the highest share of the global burden of malaria was being shouldered by sub Saharan Africa. In the year 2015, sub Saharan Africa reported around 90% of malaria deaths and 88% of malaria cases (WHO, 2016). In the year 2017, there was an estimated 219 million malaria cases whereby the ten highest burden African countries reported about 3.5 million more cases of malaria comparing to the cases reported in 2016. In Africa, malaria disease continues to threaten the lives of close to half a million people annually, whereby children under five years are the most vulnerable of this preventable and curable disease (WHO, 2018). Despite the fact that there was a reduction of estimated 20 million malaria cases in the year 2017 as compared to 2010, data collected during the period of 2015-2017 did not show any significant progress in the reduction of global malaria cases (WHO, 2018). In sub-Saharan Africa the burden of malaria disease is attributed to the human population that is expanding, weakness of the health care systems, presence of the mosquito vector and ideal environmental conditions for transmission of malaria (Killeen et al., 2004).

There are approximately one hundred and twenty species of *Plasmodium* but only five have the capacity to infect humans. These *Plasmodium* species include *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium knowlesi* and *Plasmodium ovale* (Carter & Mendis, 2002; Cox-Singh et al., 2008). The five *Plasmodium* species are found in different geographical regions. For example, 80% of the cases of malaria disease reported in sub-Saharan Africa were caused by *Plasmodium falciparum* while *P. vivax* was responsible for over 95% of malaria cases in Asia (Carter & Mendis, 2002). In regard to the five *Plasmodium* species responsible for human infections, *Plasmodium falciparum* has been reported as the most pathogenic with manifestions such as severe malaria anemia and cerebral malaria.

Female *Anopheles* mosquitoes are the exclusive vectors of malaria in mammals since they feed on mammalian blood and in the process they transmit malaria through their bites. Out of about five hundred recognized species, only approximately thirty are important vectors of human malaria while the rest of the anophelines have little consequences to the health of the public (Collins & Paskewitz, 1995). Malaria disease impedes economic development in several ways which include reduced quality of life, reduced fertility which relates to abortion cases and children born with low birth weights, savings and investment, worker productivity, population growth, medical costs and premature mortality (Sachs & Malaney, 2002).

In Kenya malaria disease still remains a major public health problem accounting for an estimated 16% of all out-patients consultations (KNBS, 2017). The probability of children under five years dying per 1000 live birth was 46 cases in Kenya (WHO, 2015). The inception of the Malaria Operational Plan (MOP) for the year 2018, showed in detail how the plan would be implemented in Kenya and this was to be based on the strategies as indicated in the President's Malaria Initiative (PMI) and National Malaria Control Program (NMCP). PMI's goal was mainly to ensure that mortalities that related to malaria reduced by 50% especially in the regions classified as high-burden countries in sub-Saharan Africa. This was to be achieved through the use of effective malaria disease treatment and prevention measures. These involved the use of indoor residual spraying, accurate diagnosis, insecticide-treated mosquito nets and treatment with artemisinin- based combination therapies (ACT). The outcome of this strategy is that it has led to reductions in mortality rates among children who are below five years of age (PMI, 2018).

Anophelines mosquitoes are quite anthropophilic and they are able to exploit habitats of different kinds that are indirectly or directly created by human beings during their different activities such as mining, farming and construction of roads or buildings. The breeding sites range from permanent water collections in rice farms and irrigation canals, water collected in ground soil depressed by livestock, temporary sun-lit pools and ditches that arise from construction (Sinka et al., 2010). The Anophelines have the capacity to exploit and adapt to varied larval habitats and this can be explained by their distribution in many geographical sites having different environmental conditions in tropical Africa (Budiansky, 2002; Lanzaro et al., 1998). *Anopheles gambiae* mosquito vectors of malaria occur in rural areas which are characterized by less pollution as compared to the urban areas which are characterized by pollution and scarce breeding sites due to high population (Coluzzi, 1993). However, there is

increased transmission of malaria in towns and urban agricultural settings and they have been shown to represent areas of vector explosion and transmission of malaria disease (Afrane et al., 2004; Matthys et al., 2006; Robert et al., 1998; Trape & Zoulani, 1987). Urbanization has created new breeding sites and *Anopheles* mosquitoes have adapted to these breeding sites and therefore their ecology differs from that in the rural areas. With increased road construction activities and urbanization means that there are changes of stream ecology that is as a result of heavy metals pollution that arise from non-point sources. However, recent information regarding heavy metal contamination relates to point and downstream activities that arise from sources that are known (Sekabira et al., 2010; Zvinowanda et al., 2009).

There have been increased levels of the pollutants in the natural environment that are released from industrial activities and domestic activities and these compounds mostly accumulate in stagnant water bodies and in rivers (Biney et al., 1994). Most of the compounds released in the larval habitats are highly toxic but *An. gambiae* has been able to circumvent the sting of the toxicants albeit at a significant biological cost. Biological cost of the mosquito relates to larval and pupal survivorship, adult emergence, reduced egg viability, fecundity, increased population doubling times, instantaneous birth and reproductive rates (Mireji et al., 2010b; Nchoutpouen et al., 2019). Heavy metals that are found in water collections are likely to arise from anthropogenic or natural sources. Heavy metals released from anthropogenic activities in most cases exceed inputs from natural sources and this has deleterious effects to the aquatic life. The transition of mosquito from rural to urban areas relates to complex interactions of biological and environmental determinants of vector adaptation to polluted urban habitats (Reed et al., 2003). Indeed, rapid population growth, unplanned urbanization and the resulting increase in pollution are rapidly changing the environment over most parts of Africa, impacting the bio-ecology and diversity of its endemic fauna with major implication on the malaria disease and other diseases (Donnelly et al., 2005; Hay et al., 2005; Robert et al., 2003). The fact that there has been urbanization which is rapid and unplanned in the cities of sub-Saharan Africa that greatly impact natural ecosystems, the changes have been observed to favour the anophelines and there has been adaptation of the anophelines to various xenobiotics which include heavy metals and they have also expanded their niche to inhabit habitats that are polluted (Awolola et al., 2007; Djouaka et al., 2007; Mireji et al., 2008).

Industrial activities and unorganized expansion of urban areas that go hand in hand with improper waste management significantly lead to polluted environments and there is also a risk of municipal waste accumulation (Mungai & Wang, 2019). Most of African cities are characterized by improper solid waste and harmful waste disposal regulations and such waste is likely to be radioactive or toxic (Kimani, 2007). These unplanned cities demonstrate problems in waste management that are characterized by uncontrolled garbage heaps, refuse that is littered in the roadsides; toxic waste that is inappropriately disposed, rubbish that has blocked streams and also disposal sites near residential areas that pose health hazards to people (Ayeni et al., 2017). Heavy metals such as chromium, aluminium, lead, zinc mercury, nickel and cadmium in the environment have been reported and they mainly arise from modern agricultural activities and industrialization (Atafar et al., 2010). Heavy metals Pollution have led to chronic and acute effects on insects which has been characterized in the form of decreased hatchability, growth of inhibition, reduced reproduction rates and developmental abnormalities (Azam et al., 2015; Mireji et al., 2010b; Singh & Heer, 2017).

Cadmium heavy metal is one of the hazardous compounds on the European Union list of harmful substances and it is a non-essential and toxic heavy metal (Waters et al., 2008). There has been a lot of concern due to incresed cadmium accumulation in the enviroment that arises mainly from anthropogenic sources (Kinuthia et al., 2020). The location of cadmium in the periodic table is between Zinc (Zn) and Mercury (Hg) and its behaviour is chemically similar to Zn. In the earth's crust cadmium exists at approximately 0.1 ppm (Wedepohl, 1995) and is usually found in impurities of Lead or Zinc deposits. The production of cadmium is mainly as a by-product during the smelting process of Lead or Zinc. Cadmium exists as a divalent cation (e.g., $CdCl_2$) that is usually a complex of other elements. The production of cadmium for commercial purposes like electroplating began around the 20^{th} century but later cadmium production is mainly for making polyvinyl chloride plastics, making nickel-cadmium batteries and paint pigments. Contamination of the atmosphere with cadmium mainly arises from fossil fuel combustion, municipal waste incineration and smelting of non-ferrous metal ores (Nordberg et al., 2007).

Cadmium contamination in humans has been classified as a carcinogen in that its toxicity has been observed on the respiratory system, kidney and the skeletal system (WHO, 1992a). Cadmium in the environment is usually present at minimal quantities but the levels have

increased due to human activities like electroplating (WHO, 1992b). It has been observed that cadmium has the capacity to travel by atmospheric transport to far places from the emission site (WHO, 2007). Cadmium accumulates in living organisms notably crustaceans, insects and mollusks (WHO, 2010). A significant source of cadmium contamination can be from municipal sewage waste that is applied to cultivation fields (WHO, 1992b). Cadmium chloride toxicity is deleterious to living organisms and its negative manifestations include reduced fecundity, high mortality rates, problems in mating, reduced longevity and reduced ability of hatching in most arthropods and insects (Luo et al., 2019; Singh & Heer, 2017). Other negative effects of cadmium at the cellular level involve production of reactive oxygen species and also being involved in oxidative stress and this causes various functional and structural changes that are characterized as lipid peroxidation (Korsloot et al., 2004), instability of the lysosomal membrane (Breackmann et al., 1999), decreased activity of the enzymes involved in detoxification and antioxidative fuctions (Augustyniak et al., 2009; Kafel et al., 2003; Lijun et al., 2005; Wilczek et al., 2004) and depletion of glutathione (Augustyniak et al., 2005).

Studies involving *Chironomus tentans* and *Baetis thermicus* aquatic insects exposed to chronic concentrations of heavy metals demonstrated adaptation to the metals they were exposed to (Clements & Kifney, 1994; Hare, 1992; Krantzberg & Stokes, 1990; Suzuki et al., 1988; Wentsel et al., 1978) and also bioaccumulation was observed (Suzuki et al., 1988). Tolerance to heavy metals depends mainly on the concentration of the specific metal and also on the type of the metal applied (Rayms-Keller et al., 1998). Heavy metal tolerance at the molecular level is usually achieved by expression of repair and defense proteins which include heat shock proteins (HSP) (Beyersmann & Hechtenberg, 1997; Kim et al., 2000; Liao & Freedman, 1998; Mireji et al., 2010a; Shimizu et al., 1997; Stohs et al., 2001), p53, glutathione, immediate early genes, metallothioneins and a spectrum of potential metal responsive proteins (Mireji et al., 2006). However, the adaption to heavy metals potentially results in significant loss in biological fitness in the mosquito (Mireji et al., 2010b).

1.2 Statement of the Problem

Anopheles gambiae is known to proliferate in clean environments devoid of pollutants. However, recent findings have established that the vector has expanded its niche to habitats polluted with organic wastes, sewage and heavy metals. This potentially expands the sources of

explosion of mosquito vector populations, mostly in the urban areas where there is poor drainage and waste disposal from industries. The prevalence of mosquito vector of malaria and its role in the transmission of malaria then translates to an impediment in economic development and increased malaria burden of the disease especially in the urban environments. The underlying molecular processes mediating adaptation of the mosquito to polluted environments are not well understood. Therefore, this study was undertaken to identify transcripts and proteins differentially expressed to enhance the survival of *An. gambiae s. s.* larvae in a cadmium heavy metal environment. Previous studies focused on establishing baseline levels of heavy metals, including cadmium, in *An. gambiae s. s*. natural habitats, expression profiles of selected genes and the associated biological costs of adaptation to habitats polluted by heavy metals. These findings formed the foundation upon which the current studies were established. The knowledge obtained from this study is critical in elucidating the molecular adaptation of the mosquito and also generation of information that is useful in designing more effective vector control methods that match changes in vector dynamics.

1.3 Objectives

1.3.1 General Objectives

To determine molecular processes mediating the adaptation of *An. gambiae sensu stricto* to cadmium heavy metal exposure.

1.3.2 Specific Objectives

i To establish and maintain a colony of cadmium treated and control untreated *An. gambiae s. s.*

ii To determine cadmium metal responsive transcripts that mediate adaptation of *An. gambiae s. s* larvae to cadmium heavy metal exposure.

iii To determine proteomic profiles (potential biomarkers) in *An. gambiae s. s*. larvae associated with exposure to cadmium heavy metal.

1.4 Hypotheses

i There is no colony of cadmium treated and control untreated *An. gambiae s. s* that requires establishment and maintenance.

ii There are no identified cadmium metal responsive transcripts mediating adaptation of *An. gambiae s. s.* larvae to cadmium metal exposure.

iii There are no identified proteomic profiles (potential biomarkers) in *An. gambiae s. s.* larvae associated with exposure to cadmium heavy metal.

1.5 Justifications of the Study

Malaria has predominantly been a disease of the rural areas in Africa and consequently rare in highly populated urban areas due to proclivity of the *An. gambiae* vector for proliferation in relatively clean aquatic habitats. However, the mosquito has been found proliferating in aquatic habitats with environmental pollution including heavy metals. This is contrary to the long held dogma that indicates their exclusive existence in clean habitats. This adaptation means that mosquito larvae must have undergone physiological adjustments, underpinned by molecular processes at various levels that have facilitated this niche expansion. Such adjustments or tolerance to heavy metals have previously been documented to lead to a reduction in the biological fitness of the mosquito that could ecologically impact the performance of the vector. The molecular processes mediating this adaption are poorly understood and yet hold key to understanding how this evolutionary process in the mosquito has impacted malaria transmission. The polluted habitats have traditionally been overlooked in vector control initiatives and this could lead to explosion of vector populations within and between seasons.

Cadmium heavy metal was selected for this study because previous studies have established that this heavy metal is highly toxic as a pollutant. Cadmium has been observed to be more toxic up to 20 times as compared to other heavy metals and on the European Union list hazardous compounds it has been ranked as the sixth in the list. Cadmium also has numerous detrimental effects on living organisms such as insects, mollusks and crustaceans including humans. This study therefore unraveled, differentially expressed transcripts and proteins that facilitate the tolerance of *An. gambiae s. s.* in cadmium heavy metal polluted environments and thus provides insights on the adaptation mechanism in the mosquito. In addition, understanding the molecular biology of the malaria vectors thriving in polluted urban environments forms the first steps towards designing more effective vector control tools that match the changes in vector dynamics.

1.6 Scope and Limitations

This study was carried out on *An. gambiae s. s.* mosquitoes reared in the insectary at the International Centre of Insect Physiology and Ecology (ICIPE). One population group was subjected to cadmium heavy metal treatment and the other population which comprised of the control group was not subjected to cadmium heavy metal treatment. These populations were raised in triplicates and third instar larvae were used in the study. The study had earlier been proposed to use cadmium, copper and lead heavy metals, however, this study was limited to assessment of cadmium heavy metal because it has been documented as one of the most toxic heavy metals having detrimental effects on organisms like insects, mollusks and crustaceans.

CHAPTER TWO
LITERATURE REVIEW

2.1 *Anopheles gambiae* Species Complex

There are approximately five hundred known species of *Anopheles* mosquitoes and out of them there are about thirty species that can transmit malaria disease. The other species of anophelines have no consequences that are of public health concern (Collins & Paskwetz, 1995). For human malaria vector to be highly efficient in the transmission of malaria disease there are traits that are needed and these include physiological competence of the vector to parasite infection, the population density should be high and also the vector should have a strong preference for human blood (White et al., 2011). There are five chromosomal forms which are Bamako, Mopti, Forest, Bissau and Savanna of *Anopheles gambiae s. s*. (Figure 1) whose naming was based on the non-Linnaean nomenclature. Each of these forms has arisen based on the inversions of chromosomes observed specifically on chromosome-2. Ecological factors like breeding sites and aridity greatly influenced the pattern of their geographical distribution (Coluzzi et al., 1985; Toure` et al., 1998). Several investigations have been carried out to determine the differences in behavior and ecology between the Savannah and the Mopti forms. Studies have so far established that the Savannah form which is the most wide spread form predorminantly inhabits temporary habitats in Savannah areas in Africa while the M form have been found to be well adapted to breeding sites that are permanent like rice fields (Diabate` et al., 2005). There are six main vectors of malaria disease in Africa out of which three belong to the *Anopheles gambiae* complex and these are *An. arabiensis*, *An. gambiae* and *An. colluzi* (Sinka et al., 2012). There are eight cryptic species that make up the *An. gambiae* complex (Coetzee et al., 2013; Davidson, 1962; Davidson & Hunt, 2013) and they ecologically differ in various ways which relate to their feeding behavior, their choice of breeding sites, their role in transmission of malaria disease and their preference for hosts (Davidson 1962; White et al., 2011). *An. gambiae* complex is widely distributed geographically through out tropical Africa and this can be attributed to its high anthropophilic nature and also its ability to colonise different habitats that range from water collections in grounds depressed by livestock, temporary sun-lit pools of water, water in irrigation canals and ditches (Budiansky, 2002; Lanzaro et al., 1998).

Africa is home to over one hundred and forty *Anopheles* species and out of them there are about eight species which are effective malaria vectors (Gillies & Coetzee, 1987; Gillies & de

Meillon, 1968). *An. gambiae s. s* and *An. arabiensis* belong to the *An. gambiae* complex and they are recognized as the vectors that are most effective for human malaria transmission (White, 1974). There are other species of the *An. gambiae* complex that are recognized and they include *An. merus, An. quadriannulatus, An. melas, An. quadriannulatus* B and *An. bwambae. An. bwambae* has been found in Semliki forest in Uganda and its breeding sites are in the mineral springs (Coluzzi, 1984). *An. melas* and *An. merus* mainly colonise salty water habitats and they have been found to be distributed mainly along the western and eastern coasts of Africa respectively. *An. quadriannulatus* are found in south-east Africa (Coluzzi, 1984) and *An. quadriannulatus* B which are found in Ethiopia are zoophilic and they have no role in human malaria transmission (Coluzzi, 1984).

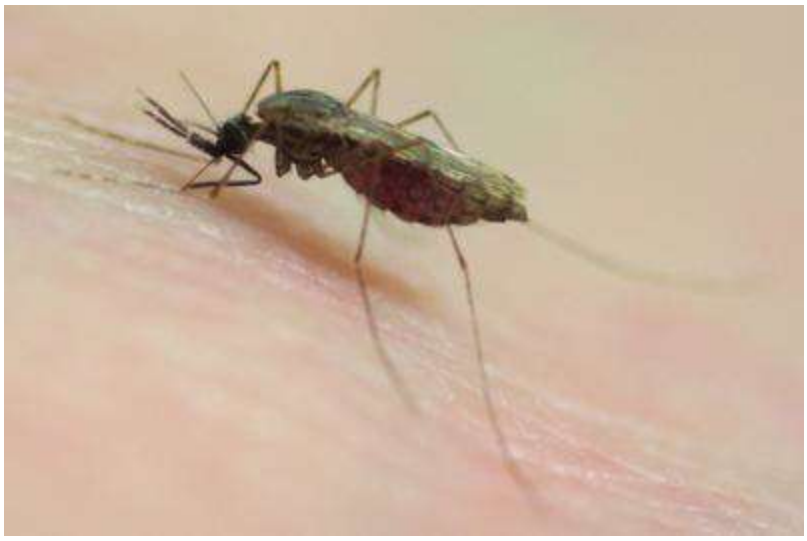


Figure 1: *Anopheles gambiae,* malaria vector

(http://www.metapathogen.com/mosquito/anopheles/)

2.2 The life cycle of the Mosquito vector

The life cycle of the mosquito vector of malaria is summarized in Figure 2. There are four stages in the development of the mosquito. The stages are egg, larva, pupa and adult as explained below.

Egg

Mosquitoes lay their eggs in water collections that offer habitats for the development of the larval stages and these could be in ditches, unused tyres where there is stagnant water. The mode of egg-laying is dependent on the mosquito type whereby there are mosquitoes that lay

their eggs singly while others lay eggs in clusters on the water surface. Initially, when the eggs are laid they are white but afterwards they become dark brown to black (Service, 1980). The shapes of the eggs are mainly boat-shaped and a few inches long. Eggs are able to hatch within two to three days when the water collections have a temperature range of about 25-28°C and at the same time there are cases when eggs can remain dormant for several months during dry conditions (Bayoh & Lindsay, 2004).

The larval stage

This is the second stage in the development of mosquitoes whereby the eggs hatch into wigglers or larva. The larva has to undergo four instar stages that characteristically differ in their sizes, before they can progress to the pupal stage. The larva lives exclusively in water feeding on organic debris, microscopic plants and animals and in some cases feed on larvae belonging to other species of mosquitoes. They use their mouth parts to filter food particles. The maturity of the larvae takes about four to ten days and this is dependent on the availability of food and also the prevailing temperatures. To enhance breathing, the larvae positions itself in a way that allows the air tube at the abdomen to project through the water surface inorder to breath but for the *Anopheles* larva it usually lies against the water surface.

The pupal stage

The pupae (tumblers) are non-feeding and this stage is refered to as the most inactive stage in the development of mosquitoes transitioning between larva and adult. Their movement is minimal unless disturbed and in most cases they lay at the water surface and this enhances their breathing through the air tubes. Depending on the mosquito species and the prevailing temperature, the pupal stage lasts about one to ten days.

The adult stage

This is the last stage in the development of mosquitoes and the adults are characterized with long legs and wings with an elongated abdomen. The distinguishing characteristics between the male and female mosquitoes are that the antennae of the males are featherly while those of the females are short with sparse hairs. The diet of the male mosquitoes mainly comprise of plant juices and nectar. The females also feed on plant juices and nectar to get energy they require for

their development. However, for the females to produce eggs they require a protein diet and therefore they feed on blood from mammals (Gillies, 1955). The male mosquitoes usually emerge before the emergence of the females (Kettle, 1992) and the males are found resting on vegetation near the site of emergence awaiting the emergence of the females. Soon after female emergence, mating takes place and the eggs are fertilized. These can later be laid and the cycle is repeated (Kettle, 1992).

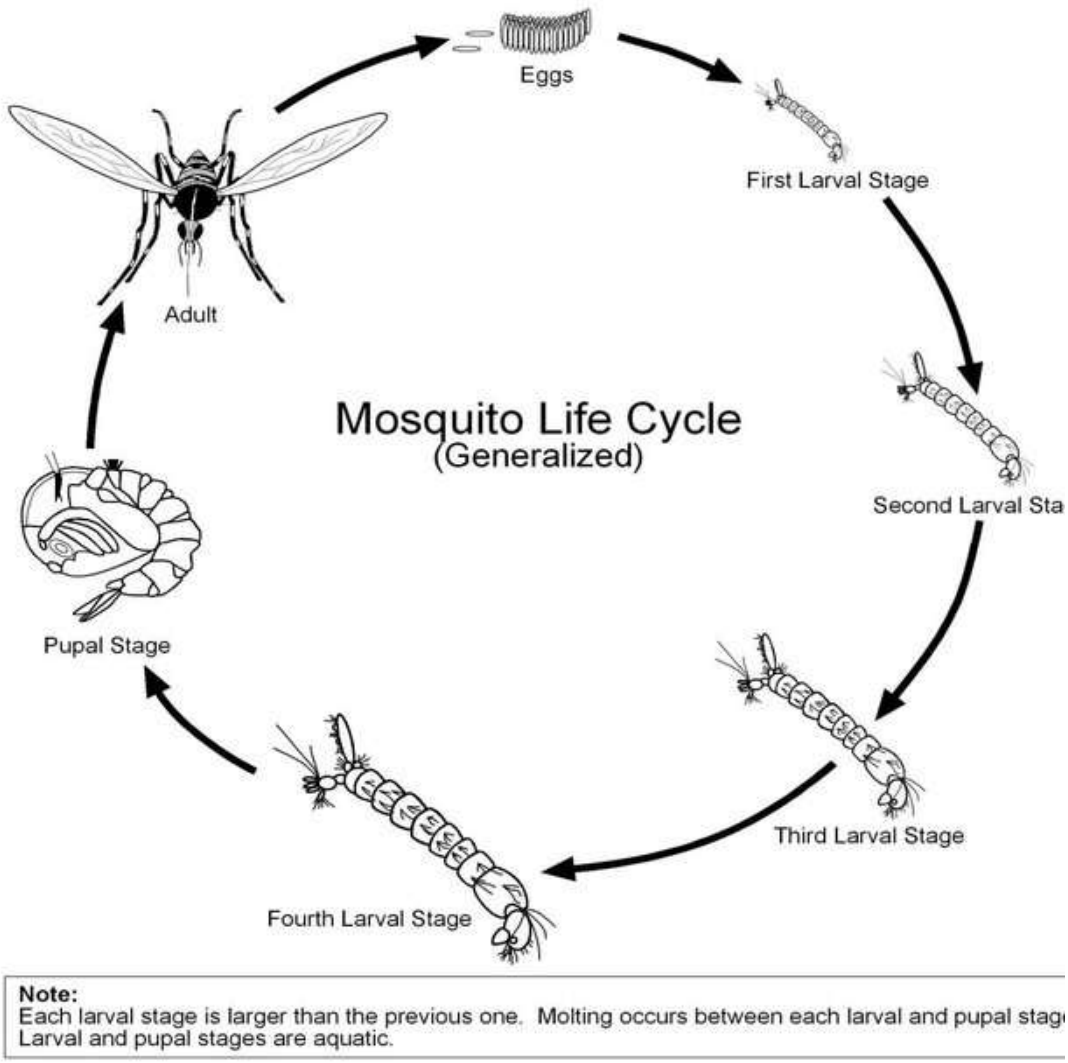


Figure 2: The life cycle of a mosquito. (www.mosquitoes.org/Lif)

2.3 Malaria parasites

Approximately one hundred and twenty *Plasmodium* species have been identified and out of these only five are causative agents of malaria disease in humans. The others cause infections

in reptiles, birds and other mammals. The five *Plasmodium* species are *P. malariae, P. ovale, P. knowlesi, P. falciparum and P. vivax.* Research has shown that *P. ovale* consists of two species (Duval et al., 2009). *P. falciparum* which is predominantly found in tropical Africa is one of the most deleterious species that accounts for most of the serious malaria cases observed in expectant women and children under the age of five years and it accounts for the largest number of deaths reported in Africa (Bozdech et al., 2003; Breman et al, 2007; Snow et al., 2005). Initially, *P. falciparum* was reported as an exclusive human malaria parasite but this has been disputed and it has been reported that *P. falciparum* is closely related to a chimpanzee parasite known as *P. reichenowi* (Prugnolle et al., 2010). The other *Plasmodium* species do not cause severe clinical manifestations of malaria disease.

2.4 The life cycle of malaria parasites

The life cycle of the five *Plasmodium* species that are responsible for malaria disease in humans share the same life cycle as shown in figure 3. The cycle begins with a bite from a female mosquito that harbors sporozoites that are infective and they are introduced into the mammalian blood system. The infective sporozoites take a few minutes in the blood system and phagocytes destroy most of the sporozoites. The remaining sporozoites penetrate the liver parenchymal cells where asexual multiplication takes place and this is refered to as the pre-erythrocytic schizogony. After asexual multiplication, a large schizont that is unpigmented results and this contains merozoites that attack the erythrocytes once released in the blood system. In the blood stream, merozoites get attached to erythrocytes and through a parasitophorous vacuole the merozites are invaginated into the red blood cell and here there is deposition of haemozoin pigment (Aikawa, 1980). The merozoites that have been ingested get transformed into trophozoites and when they are fully developed, asexual multiplication takes place forming a schizont and this is refered to as the erythrocytic schizogony that produces a few merozoites (Aikawa & Seed, 1980). An attack of malaria is felt when merozoites are released and this cycle is repeated and other erythrocytes are continually invaded. Cycles of schizogony are repeated with no division of the trophozoites but they are transformed into gametocytes whose development does not take place any longer in a mammalian host but keep circulating in the blood system awaiting a female *Anopheles* mosquito to take up the gametocytes when sucking blood from the infected host (Kettle, 1992). When the microgametocytes are taken up by

the *Anopheles* mosquito they undergo exflagellation process becoming microgametes. The microgametes are the female gametes and they fuse with male gametes or macrogametes forming a zygote. The zygote formed does not move for about one day but later it elongates forming ookinetes that exhibit motility (Aikawa & Seed, 1980; Kettle, 1992). The motile ookinete have the ability to penetrate mosquito's midgut wall thereby forming oocysts through the process of sporogony. The formed oocysts later become enlarged into sporozoites that are motile and when the wall of the oocyst burst, infective sporozoites are released and they move to the mosquito's salivary glands awaiting to be injected to a human host during the next blood meal. The cycle is then repeated.

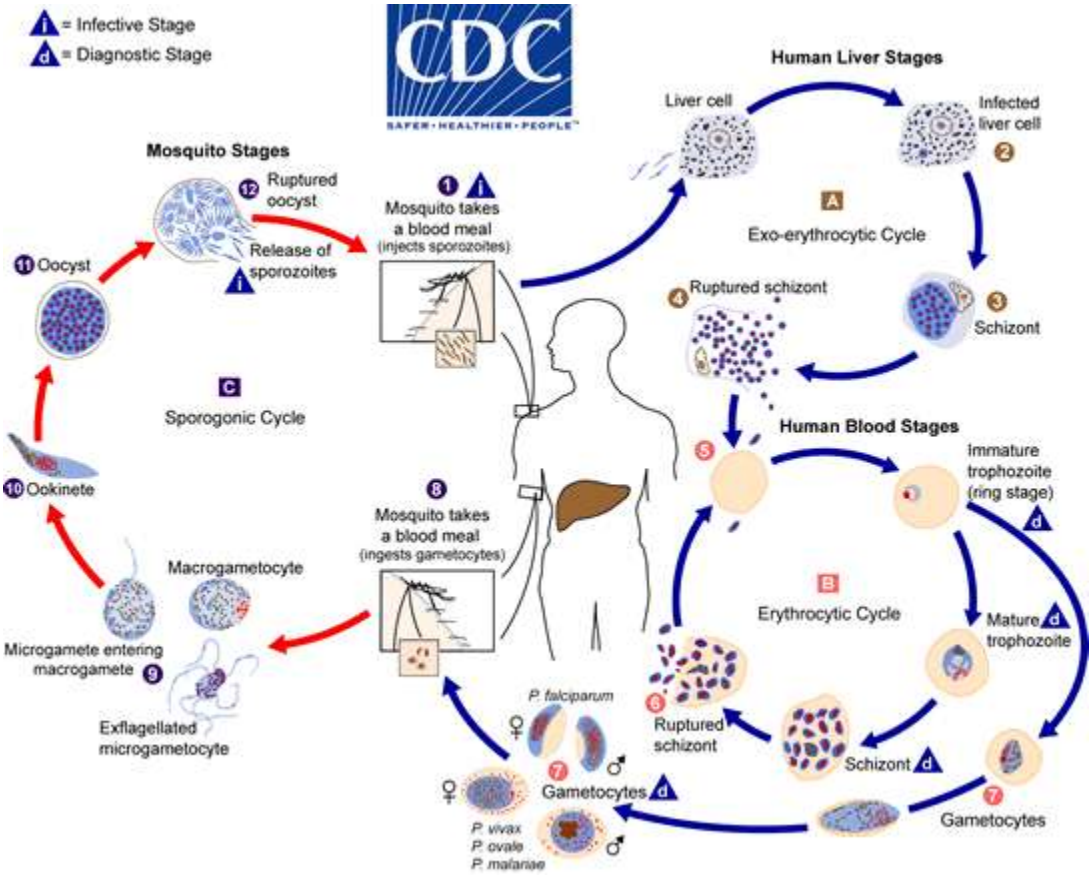


Figure 3: The life cycle of *P. falciparum,* adopted from the Centre for Disease Control (CDC) website *(http://www.dpd.cdc.gov/dpdx/HTML/Malari a.htm)*

2.5 Clinical manifestations of *P. falciparum* infection

P. falciparum infections in areas endemic for malaria disease are characterized by headache, fever, lethargy and malaise and in most cases recovery is often achieved without the use of any drugs. When individuals are repeatedly exposed to malaria disease their immunity build up thus conferring protection against the disease even though there are minimal infections that occur without exhibiting any clinical symptoms (Warrell et al., 1990). Most of the malaria deaths can be attributed to cerebral malaria that mainly manifests through a coma that is combined with convulsions. However, where there is impairement of consciousness of whatever level mostly points to involvement of the cerebral area of the body (Warrell et al., 1990). Another clinical manifestation of infection with *P. falciparum* is severe anemia that mainly arises from a reduction in the erythropoiesis process and also destruction of red blood cells (RBC) (Menendez et al., 2000). Expectant mothers suffer a high risk of malaria infection with *P. falciparum* and the risk has been shown to be higher in the initial pregnancy as compared to the subsequent pregnancies (Brabin, 1983; McGregor, 1984). During pregnancy complications that arise due to infections of malaria relate to anemia resulting in low birth weights, abortions and also high infant mortalities (Granja et al., 1998; McGregor, 1984). *P. falciparum* complications in expectant women that lead to anemia in infants and reduced weights by the time of birth has been shown to arise from parasites sequestrating in the placenta spaces through the use of chondroitin sulphate A (CSA) receptor in the placenta (Hviid & Salanti, 2007; Rogerson et al., 2007). Other clinical manifestations of malaria disease are fluid retention that leads to distress in the respiratory system (English et al., 1996; Warrell et al., 1990), severe hemoglobinuria resulting from hemolysis in the intravascular system and also hypoglycemia (Warrell et al., 1990).

2.6 Burden of Malaria Disease

Mosquitoes are vectors of malaria disease which threatens the lives many people in sub Saharan Africa being transmitted through bites of infective female *Anopheles* mosquito (de Castro et al., 2006). Malaria infections that are caused by *P. falciparum* are normally characterized by symptoms like chills, fever and anemia and in severe cases malaria usually result in a coma and in the worst cases the infections result in death (Coluzzi, 1992; Collins & Besansky, 1994; Mbogo et al., 2003). The burden of malaria disease have been experienced

through out the world as evidenced by malaria infections and deaths reported over most parts of the globe (Hay et al., 2004; Hay et al., 2005; Snow et al., 2005; WHO, 2018). In the year 2017, a total of 87 countries reported over 200 million malaria disease cases and deaths numbers of close to half a million people as a result of malaria infections. The burden of malaria disease is greatest in Africa with reports indicating that the African region had 92% and 93% of malaria cases and death cases respectively. In the attempts to control and curb malaria in the countries that are endemic, approximately US$3.1 billion was released which represented about 28% of the total funding for the control and elimination of mosquitoes (WHO, 2018). The African continent shoulders the highest burden of malaria disease since it is home to the malaria vector *An. gambiae* and this in turn translates to poverty incidences that is occasioned by the economic burden of malaria disease (Sachs & Malaney, 2002). The intensities and the transmission rates of malaria disease differs depending on the seasons in the different countries and this thereby means that vector abundance also differ during the varied seasons in the endemic regions (Greenwood et al., 2008).

2.7 Mosquito Larval Habitats

The habitats for *An. gambiae* larvae are clean water collections that mostly emanate from activities that are undertaken by humans and animals. The human activities span from agricultural activities like digging trenches, irrigation ditches, burrow pits and drilling of boreholes and these activities go along way in creating breeding sites for larvae production (Sinka et al., 2010). Other human activities involved in formation of mosquito larval habitats are road construction activities that form roadside puddles from tyre tracks and creation of artificial water bodies like dams. Animals also participate in creation of larval habitats by their hoof prints that form depressions in the soil and thereby serve as water collection points during the rainy seasons thus enhancing the mosquito larvae to thrive (Gimnig et al., 2001; Minakawa et al., 1999). *An. gambiae* larvae breeding sites also include lagoons, shores of lakes and on river edges. There is a growing evidence of niche expansion of the vectors of malaria disease to urban environments that are highly polluted with wastes from industrial activities whose wastes range from oils that spill from oil refineries, heavy metal wastes from manufacturing industries and electroplating processes. Other wastes in the urban areas originate from blocked sewage plants that form sewage ponds, from garbage sites where there is improper disposal of wastes and

wastes from domestic activities and car washes. The change in the mosquitoes to colonise polluted urban environments could be attributed to the fact that there is little competition in these habitats and also predation is minimal in the polluted environments (Coluzzi et al., 1984). There are studies done previously that point at niche expansion of mosquitoes to polluted environments mentioned above inaddition to human wastes and drains. These include a study carried out in Dar es salaam in Tanzania (Sattler et al., 2005), Lagos and Ibadan, Nigeria (Awolola et al., 2007; Djouaka et al., 2007), Accra and Tema, Ghana (Chinery, 1984; Chinery, 1995), Kinshasa, Democratic republic of Congo (Coene,1993) and Kisumu and Malindi, Kenya (Mireji et al., 2008).

The process of urbanization and increasing human densities in the urban environments serves to reduce the larval habitats that would otherwise be used for vector breeding and also as stated earlier, urbanization greatly raises the pollution level in the urban environments which negatively affects the development and survival of the malaria vector. Overall, the ever increasing pollution in the urban enviroments leads to a reduction of the malaria vector density as a consequence of the scarcity of suitable and productive larval development sites (Robert et al., 2003; Trape & Zoulani, 1987). It is necessary to note that malaria transmissions occurs in African towns and evidence is rapidly accumulating that the urban environment might not be so reluctant to *Anopheles* development than previously acknowledged (Mireji et al., 2008). Experimental investigations have demonstrated the development of tolerance to increasing heavy metal challenge in the *Anopheles gambiae* vector to Cd, Cu and Pb for several generations in the laboratory (Mireji et al., 2008). The tolerance of the malaria vector to the urban polluted sites in Africa would have dramatic consequences for the epidemiology of this devastating disease, as the urban the population in Africa is rapidly increasing.

Previous research have established the presence of *Anopheles gambiae* larvae in polluted urban environments contrary to the long held dogma of *An. gambiae* thriving in clean water devoid of pollutants which is a common scenario in the rural settings (Sattler et al., 2005). Examples include a study carried out in the urban towns in Kenya that have indicated the presence of heavy metal pollutions in mosquito larva habitats (Mireji et al., 2008; Karungu et al., 2019). In Dares salaam city in Tanzania a study was done using *An. gambiae s. l* and it was established that this species had the capacity to breed in sewage ponds and also swamps yet these environments had a high level of organic waste water (Sattler et al., 2005). A different study

undertaken in Pakistan and Ghana also using *An. gambiae s. l* similar to the Tanzanian case study, also reported tolerance of the *Anopheles* species and its ability to breed in environments that were highly polluted and this further demonstrates niche expansions of the mosquito vector of malaria (Mukhtar et al., 2003). *An. arabiensis* have also been observed to be increasingly adapting to polluted environments with this adaptation being linked to insecticide resistance (Oliver & Brooke, 2018).

2.8 Malaria transmission

The risk of human malaria disease to the Kenyan population is about 70%. Therefore, various control strategies have been put in place to curb the menace of the disease (Mbogo et al., 1995). The transmission of human malaria disease is effective when the mosquito vector and the respective *Plasmodium* species are present (Beier et al., 1994). In Kenya the major vectors of malaria are *An. funestus, An. merus, An. gambiae* and *An. arabiensis* (PMI, 2018). These vectors vary in their abundance and distribution which is attributed to the different climatic conditions especially in relation to the varied rainfall patterns and temperatures that is usually observed in different regions in the country. For there to be a successful transmission of *P. falciparum* infection, the female *Anopheles* vector mosquito must bite a person about two times in a week. This is because the first feeding on human blood is meant acquire the gametocytes whose development does not take place in a human host but has to be taken up by the mosquito vector of malaria for further development in the mosquito. The second bite is then necessary because after the parasites have completed their developmental stages in the vector, they form sporozoites that need to be injected into the human host for transmission of the *Plasmodium* parasites to take place (Kabiru et al., 1997). This therefore means that there can be incidences that mosquito bites can fail to transmit the disease especially if the vector is not harbouring the infective sporozoites in their salivary glands (Mbogo et al., 1995).

2.9 Effects of human activities on malaria vector population

Massive research work has been directed towards various aspects of mosquito biology, vector dynamics in malaria transmission and identification of the various vectors types but there has not been focus on how the human activities have impacted the numbers or the population level of the mosquitoes. Humans interfere with the environment through various ways which

include clearing forests inorder to undertake agricultural activities like farming, keeping livestock, creation of space for development of urban centres (Norris, 2004). These transformations of the environment lead to imbalances in the ecology of mosquito vector of malaria and this has made mosquitoes to change the way the breed to the point whereby mosquito larva have been able to breed in polluted environment (Mireji et al., 2008; Patz et al., 2000). Deforestation creates new breeding sites for mosquitoes and in most cases in the African continent there has been explosion of the mosquito population. This is because water collects in ditches or furrows that result due to deforestation process and therefore larval habitats are generated (Patz et al., 2000). This then translates to outbreaks of malaria disease in such areas where initially due to the thick forest cover which is a characteristic before deforestation would not have favoured the presence of mosquitoes (Vittor et al., 2006). Elimination of the forest cover also leads to population growth because people move to those areas in search for employment in the farms and the road construction sites. Increased population later brings about the process of urbanization and with time pollution follows since the social amenities like waste disposal and sewage systems do not match with the rising numbers of the people in an area and consequently the population of the mosquitoes is affected (Norris, 2004). Agricultural activities are also characterized by creation of water bodies like dams to collect water for farming and in the process of farming, irrigation canals or paddles are formed which act as new sites for the breeding of the mosquitoes and thereby increasing the vector populations (Norris, 2004).

2.10 Methods used to control mosquito vector of malaria

The malaria disease menace has really been thought thorough by many countries that are greately affected by this devastating disease and the control of the vector population has been seen as the most viable option for reduction or better still for the eradication of the malaria disease in the endemic regions (Okogun, 2005). In the past a remarkable reduction in the mortalities and morbidities in malaria endemic areas of over 75% in about fifty five countries has been reported (WHO, 2014). Based on the progress that was made in malaria disease control by use of the vector control approach, many players that are involved in this noble task were called upon to engage in the vector control strategies that would ensure continued efforts to eliminate malaria. These like-minded bodies included the Global Technical Strategy for Malaria by the Global Malaria Programme under the World Health Organization (RBM, 2015; WHO, 2015).

The intensity of malaria disease transmission can be explained in terms of the bites that one receives from a female *Anopheles* mosquito which must be infective within a specified period of time. This intensity of malaria transmission is thus refered to as the entomological inoculation rate (EIR) (Kabiru et al., 1997). There are many models of malaria control that are based on the control of mosquito larvae and adult populations and these strategies have seen great reductions in the entomological inoculation rates and consequently a reduction in the transmissions of the malaria disease (Killeen et al., 2002). There have been many intensified campaigns that aim at promoting the vector control strategies that have been in place in the previous years. The methods that involved in the control of adult mosquitoes include indoor residual spraying (IRS) and insecticide-treated bed nets (ITNS) (Bhattarai, 2007; Fegan et al., 2007; Noor et al., 2009). Vector control mode that focuses on the elimination of the larvae involves the use of biological control agents such as *Bacillus thuringiensis israelensis* (*Bti*) (Fillinger et al., 2003).

2.10.1 Insecticides

Vector control strategies for malaria disease control is mainly focused on the reduction of adult mosquitoes through the use of the two commonly used methods and these are insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS). These methods work to lower the density of the mosquitoes and since the lifespan of the adult mosquitoes is reduced, it therefore means that even the malaria disease transmission is reduced to a significant level since the vectorial capacity is drastically reduced (Walker & Lynch, 2007). On the other hand, control of the larvae population is achieved by the use of larvicides and this indirectly reduces the malaria vector population level and consequently the malaria transmission capacity and in most cases this method has been used to complement insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) methods. Spraying on the space in the areas where mosquito density is high has also been practiced for malaria control though it is not as effective as the use of IRS and ITNs (WHO, 2011a).

The insecticides that have been allowed for the control malaria disease targeting the adults are mainly pyrethroids and (DDT) (Nauen, 2007). The mode of action of these insecticides is that they block the neuronal activity of the nerve membranes of the mosquitoes thereby causing paralysis and death of the malaria vector by their neurotoxic acivities (Burt &

Goodchild, 1974). In campaigns for the eradication of malaria in endemic areas, dichlorodiphenyltrichloroethane has been used especially for indoor residual spraying and it has been found to be effective even though there are public health and environmental risks that are associated with it (Rogan & Chen, 2005). The safety of pyrethroids is higher compared to the use of DDT and therefore pyrethroids have been extensively used in malaria disease control strategies notably in the application on the bed nets for mosquito control (Zaim et al., 2000). Despite the issues relating to environmental and public health, malaria control based on the use of insecticides remains as one of the strategies due to the fact that mosquitoes of the *Anopheles* species are still susceptible to these insecticides. However, with the increased use of insecticides and pesticides on the targeted vectors, resistance to the insecticides has been reported in mosquitoes and other insect populations (Corbel et al., 2007; Nauen, 2007; Oliver & Brooke, 2018; Protopopoff et al., 2008).

In the African continent the method that is commonly used for malaria disease control is the use of bed nets that are treated with insecticides and they are also known as long-lasting insecticidal nets (LLINs). The bed nets mode of action is that they provide a barrier between the vectors and the humans so there is minimal contact between humans and mosquitoes. In addition, the bed nets are treated with insecticides which have adverse effects on the mosquitoes since they are neurotoxic. These treated nets have found application especially in African households both for the adults and children and their use has led to significant reductions in malaria incidences in many communities especially for the risk group that involves young children and expectant mothers (Alonso et al., 1991; Magesa et al., 2005). The benefits that have been achieved by this mode of control is that malaria vector population has reduced drastically and therefore the malaria transmission rates have been minimized thus reducing the malaria disease mobidities and mortalities especially in sub-Saharan Africa (Lengeler, 2004). This has also been made possible because communities have also embraced and practised this mode of malaria control. Studies have shown that in sub-Saharan Africa the use of insecticide treated bed nets have increased tremendously as opposed to indoor residual spraying, with West Africa taking the lead (WHO, 2011b). The only disadvantage to this strategy of malaria disease control is the development of resistance in the malaria vector to the insecticides that are mainly used for impregnation of the bed nets (Greenwood et al., 2008; Ringwald, 2007).

The behavior of adult mosquitoes which comprises of landing and resting on the surfaces in homes and buildings has been exploited for vector control through indoor residual spraying which entails the use of chemicals that are long-acting to kill the resting mosquitoes (WHO, 2006). The chemicals are usually sprayed on the surfaces where the mosquitoes rest after feeding on blood from human hosts. Exposure of the mosquitoes to the toxic chemicals usually kills the mosquitoes and the outcome is that the malaria vector populations are reduced and in additional to that the transmission of the malaria disease is also reduced significantly (WHO, 2011b). There are also chemicals used for IRS that bear an additional role of being mosquito repellents that deter them from gaining access to rooms that are treated (WHO, 2006). In order for this method to bear fruits, there are factors which are to be considered which relate to well planned and executed spraying procedures putting in mind the chemicals to be used in the operation and also the fact that spraying must be done at the right time (WHO, 2011b).

2.10.2 Repellents

Repellents that are applied topically have been used to repel mosquito vectors of malaria thus preventing physical contact between the vector and humans who have undertaken a personal responsibility of applying the repellents. This procedure then protects individuals from bites of mosquitoes especially when outdoors and this ultimately leads to prevention of malaria disease (Tangena et al., 2018). Repellents have double role of killing and repelling the mosquito vector of malaria disease and therefore individuals whose work entails being out during the night or working in areas that are forested where the mosquitoes are present should undertake topical application of the repellents to keep mosquitoes at bay (Curtis, 1992; Rozendaal, 1997). The repellent of choice that has been used since 1957 is referred to as DEET (Di-ethyl 3-methyl benzamide) and it has been classified as the most effective form of repellent (Brown & Hebert, 1997; Coleman et al., 1993; Debboun et al., 2014; Yap, 1986). DEET is a long acting repellent since it has the capacity to repel mosquito vectors for a period of about nine hours and this means that application during the night can be done only once or at most twice (Tawatsin et al., 2006). Despite the effectivity of DEET that has been recorded, there are disadvantages that are attached to its use as a mosquito repellent. The disadvantages that come with DEET are firstly, the concentration level has to be around 30% so that it can be considered effective. Secondly, DEET produces a bad smell and it also has a feeling that is oily and therefore most people prefer to use

other vector control methods as opposed to the use of repellents. Thirdly, this form of repellent has negative effects on plastics and rubber by having the capacity of dissolving synthetic rubber and also plastics (Debboun et al., 2014). This has led to the development of formulations like picardin though it also has disadvantages that are related to DEET especially regarding the high concentration level which is a requirement for picardin to be effective (Afify et al., 2019; Lo et al., 2018).

Research trials and studies have shown that DEET as a repellent can be used in addition to other vector control methods and this in regard to a study carried out in a small community in India where it was reported that this mode of control offered protection to the people thereby reducing the incidences of malaria disease (Dutta et al., 2011). A repellent soap having DEET formulation was also tried in a population of refugees in Pakistan and it was also confirmed that the repellent soap offered protection against the malaria vectors from the people (Rowland et al., 2004). A study carried out in south Laos reported that the use of LLINs in conjuction with DEET lotion at a lower concentration level of 15% as opposed to a concentration of 30% provided protection against the mosquito vector of malaria and thereby led to ultimate reduction of the incidences of malaria disease in the region (Chen-Hussey et al., 2013). Another study carried out in Malaysia and Thailand reported that the biting rate of mosquitoes was reduced by about 80% when DEET repellent was used at a lower concentration of about 20% and consequently the incidences of malaria disease were reduced (Lindsay et al., 1998; Yap et al., 2000).

2.10.3 Ethnobotanical plants with phytochemicals

The substances that are present in particular plants have been exploited and used against mosquito vectors of malaria and they have also found application against other insects in addition to mosquitoes (Caraballo & King, 2014; Curtis, 1992). Studies have therefore been directed to determine specific extracts and active compounds or phytochemicals from plants of different families that can be formulated as larvicides or insecticides to be used for the control of malaria vectors (Kidane et al., 2013; Tiwary et al., 2007). It has been found that oil extracts from particular plants that are non-toxic and are environmentally friendly have been used as repellents, insecticides and in other cases as larvicides against various vectors of diseases (Caraballo & King, 2014; Oljira, 2015). In the rural areas in Africa there are plants that have been used for protection of the people from insect bites and in some cases oils have been extracted that contain

essential phytochemicals and once applied on the body they function by repelling insects (Romi et al., 2001; Weka et al., 2004). The plants have also been analyzed to determine their repellency characteristics and also their toxicity level (de Boer et al., 2010; Gou et al., 2020). The use of plants and their products or extracts have been accepted in a greater way in the rural areas and the forms that are used range from leaves and wood that are burnt producing smoke and smell that repels mosquitoes and other blood feeding insects (Bockarie et al., 1994; Gakuya et al., 2013; Kweka et al., 2008; Maia & Moore, 2011). A Kenyan example is where ethnobotanical plants leaves have been burnt using stoves or leaves placed around beds and they are used against *An. gambiae* mosquito vectors of malaria disease (Seyoum et al., 2002a; Seyoum et al., 2002b). However, as the lifestyles of people change and with increased urbanization, these traditional methods of control of mosquitoes and other biting insects are slowly being sidelined (Gonza`lez et al., 2011) and people are turning to products that are more modern in the form of neem and pyrethrum (Debboun et al., 2006). *Elaeis guineensis* plant has been studied extensively as an ethnobotanical plant and it has been found to contain repellent properties and therefore it has been used to repel insects and mosquitoes when prepared in formulations oils and lotions obtained from the plant extracts (Konan et al., 2003; Youmsi et al., 2017). The phytochemical composition of this *E. guineensis* plant has been analyzed on the leaves and flowers and it has been reported that it contains terpenoids, flavonoids and tannins among other compounds (Lajis et al., 1985; Yin et al., 2013). The phytochemicals that are present in this plant are harmful to the insects including mosquitoes and therefore the extracts from *E. guineensis* plant are feasible for the control of mosquitoes contact with humans thereby reducing the malaria disease transmission rates (Agaba et al., 2015; Carlsen & Fomsgaard, 2008; Christov et al., 1997; Golawska et al., 2006; Khanam et al., 2006; Ohsugi et al., 1985; Simmonds, 2001; Simmonds, 2003).

2.11 Control of *Anopheles* larvae in Malaria Control Initiative

The larvae of *Anopheles* mosquitoes can be targeted in the quest for malaria control in endemic regions. This can be done whereby the larval habitats are modified in such a way that the survival and growth of the larvae is compromised (Kitron & Spielman, 1989). The rationale of this approach is to interrupt the mosquito life cycle and prevent the progressive growth stages up to adult stage from taking place. This is because the adults are responsible for biting people and transmitting the *Plasmodium* parasites that cause malaria disease. The traditional method

that has found application especially in the rural areas for killing mosquito larvae entails the use of oil or paraffin that is applied on water collections containing mosquito larvae. Application of paraffin or oils causes suffocation to the larvae through lack of oxygen. The drawback associated with this mode of mosquito control is that suffocation will also occur to other aquatic organisms and end up being killed (Wigglesworth, 1976).

2.11.1 Biological control

The use of insecticides for the control of mosquito vectors of malaria disease have greatly been hampered by rise in insecticide resistance among the mosquitoes and also the fact that insecticides have adverse side effects on the environment leading to elimination of even non-target organisms. Therefore, alternative control of mosquitoes in the form of biological control has been implemented. This is mainly the use of biological agents such as fishes or predators to control the larvae of mosquitoes (Benelli et al., 2016). There are predators that have been effective in feeding on the larvae and pupae of mosquitoes and they have found application in the reduction of Culicidae populations thereby reducing the incidences of malaria disease menace (Griffin & Knight, 2012; Kumar & Hwang, 2006; Louca et al., 2009). Larvivorous fishes are fishes that feed on the mosquito larvae in their different instar stages and they have found application as biological agents for the control of mosquitoes (Chandra et al., 2008; Kamareddine, 2012; Subramaniam et al., 2015). The fishes that have been commonly used are in the Genus Gambusia and Poecilia (Chandra et al., 2008; Chobu et al., 2015; Das & Prasad, 1991; Kamareddine, 2012; Kweka et al., 2011; Moyle, 1976; Ohba et al., 2010; Walton, 2007) and they have effectively reduced the populations of mosquito larvae which translates to a reduction in the mosquito vector density and ultimately a reduction of malaria transmission rates (Chandra et al., 2008; Kumar & Hwang, 2006; van Dam & Walton, 2007). The only draw back associated with the use of larvivorous fishes is that they prey on other aquatic organisms such as amphibians which are not part of the target group of biological control (Rupp, 1996; Kats & Ferrer, 2003).

Copepods have also been used as biological agents for the control of mosquito larvae (Marten et al., 1989; Schaper, 1999; Vu et al., 1998). The rearing of copepods is cheap and the maintenance of the colonies is not labour intensive and therefore they can be mass reared to be used as biological agents for the control of mosquito larvae (Soumare & Cilek, 2011; Chitra et al., 2013). Copepods have found application in Vietnam for the control of *Ae. egypyti* larvae and

these biological agents have been effective in eradicating *Ae. egypyti* and the transmission of dengue virus have totally been eliminated (Kay et al., 2010; Sinh et al., 2012; Vu et al., 2005). The use of copepods as biological control agents of mosquitoes is limited by the fact that different mosquito species harbour different habitats and copepods are not effective in all kinds of habitats therefore their efficiency is achieved in specific habitats (Hales & van Panhuis, 2005).

There are *Bacillus* strains that produce toxins that have larvicidal properties and they have been exploited as biological agents for the control of mosquito larvae (Lacey & Undeen, 1986). These species include *Bacillus thuringiensis israelensis* H-4 and *B. sphaericus.* In the European countries *Bacillus thuringiensis var. israelensis (Bti)* has found application as a larvicidal agent in the control of *Ae. Aegypti* populations (Armengol et al., 2006; Novak et al., 1985) and also the control of *Ae. Albopictus* larvae (Lam et al., 2010). However, there is a likelihood of development of resistance in *Aedes* mosquito to the toxins released by *Bti* (Georghiou & Wirth, 1997). Other biological agents that have been used in the control of mosquitoes include the use of amphibians (Bowatte et al., 2013; Brodman & Dorton, 2006) and also juvenile stages of the odonate organisms and water bugs have also been used for biological control (Bailey, 1989; Cloarec, 1990; Shaalan et al., 2007; Singh et al., 2003; Venkatesan & Jeyachandra, 1985). In addition, there have been incidences where larvae of other species of mosquitoes have been used as biological agents in the control of mosquito vectors of malaria disease (Focks et al., 1985; Steffan & Evenhuis, 1981).

2.11.2 Chemical larvicides

The use insecticides that target the adult stage of mosquitoes in some cases have been inffective or have been costly and therefore chemical larvicides have been used as an alternative. The larvicide, paris green dust have been used in controlling mosquito larvae though its use comes at a cost since copper level is high hence negatively affects the environment because of the toxicity associated with copper metal (Service, 1986). The limitations that come along with the use of larvicides are their toxicity that has adverse effects to aquatic organisms and also the fact that they are resistant to degradation having the capacity to persist in the environment thereby leading to environmental pollution (Charlese et al., 1995). Other chemicals that have been used as larvicides include lindane, dieldrin and DDT (WHO, 1993), but in cases where

there are larvae strains that are resistant to the chemicals then an alternative chemical like parathion can be applied (Metcalf et al., 1962).

2.12 Genetic control of mosquitoes

The high reproductive rates and the flexibility of the mosquito vectors genome have hampered malaria vector control methods. In addition, there is also development of resistance to the insecticide classes that have found application in the control of mosquito vectors of malaria (Nicholson, 2007). The use of chemicals in vector control approaches also suffers draw backs in terms of the toxicity to the environment associated with the chemicals and also mortalities of the aquatic organisms that were not targeted in the control of mosquitoes (Knols et al., 2006). Another compounding issue is that there are species that are closely related and they end up forming cryptic species that undergo speciation inorder to adapt to the environment that is progressively changing due to human activities (Benedict & Robinson, 2003). Therefore alternative vector control approaches of genetic control of mosquito population have been attempted. Several methods are applicable for genetic control strategies and these include the use of the sterile insect technique, Wolbachia and gene drive approaches among others.

2.12.1 Sterile Insect Technique (SIT)

The concept of sterile insect technique (SIT) was developed against the screw worm in the year 1950 and 1960 (Klassen & Curtis, 2005). This technique has proved successful in the eradication of screw worms and tsetse flies from Ugunja Island in Zanzibar (Vreysen et al., 2000). SIT is pest management method that has no adverse environmental effects which entails production of large numbers of males of the insects of interest that are then irradiated so as to form mutations that are lethal in their sperms. After irradiation the male insects that are now sterile are allowed to mate with non-irradiated females present in that particular area and the end result is that there are no progenies that are produced (Wilke & Marrelli, 2012). When this process is repeated over a period of time there will be drastic reduction of the target insect populations (Wilke et al., 2009). The advantages of this technique are that male insects are species-specific in their search for their female counterparts therefore population reduction is observed where this technique has been applied. In addition, this technique is species-specific and does not introduce foreign genetic substances in the environment (Robinson et al., 2002) and

also based on the fact that male mosquitoes are the ones that are released and not the female mosquitoes, the biting rates of the mosquitoes are not increased in the area where this technique is being applied (Wilke & Marrelli, 2012). The major draw back of this technique is that the performance of the males that have been irradiated is normally reduced (Bellini et al., 2013b). For the SIT to be effective, other vector control methods must be put in place to reduce the population level of the target group and then later the irradiated males can be released. Another disadvantage is that sex sorting must be done before implementation of the SITand this process is tedious and there can be incidences where female insects can escape and they will be potential vectors of malaria disease. Also the release of sterile males should be carried out repeatedily to ensure that there are high numbers of sterile males in the wild available for mating with the wild females and therefore SIT should aim producing irradiated males that are equally competitive and fit in relation to the wild male population (Atyame et al., 2016; Bellini et al., 2013a).

2.12.2 *Wolbachia*

The endosymbiotic bacterium *Wolbachia pipientis* has been found to be infective to almost half of all species of insects (Hilgenboecker et al., 2008; Zug & Hammerstein, 2012). The transmission is usually vertical through the host egg and there is manipulation through the induction of cytoplasmic incompatibility (CI) by the strains of *Wolbachia* of the reproductive system of insects. This leads to reproduction being enhanced in the infected females as opposed to the uninfected females. Females that are infected with *Wolbachia* strains spread *Wolbachia* in the entire population because they are able to mate with males that are infected and those that are not infected at all. When male mosquitoes infected with *Wolbachia* are released into wild that has uninfected females, the females acquire the infection and they are all sterilized and this is because *Wolbachia* has the capability of spreading in the entire population (Flores & O`Neill, 2018). The rationale behind the use of *Wolbachia* strains in the control of vector-borne diseases is that infected females lack the capability to spread the parasites to human beings and therefore there is a reduction in the disease incidences and in the long run there total elimination of the disease in the areas where this technique has been implemented (Dorigatti et al., 2017; Ferguson et al., 2015). The advantages of *Wolbachia*-based mode of reduction of disease transmission in mosquitoes include the number of infected mosquitoes released in the wild population are lower as compared to other population suppression methods like SIT, the *Wolbachia* strains are usually

persistent in the population and they continue spreading across the population such that after a period of time almost the entire population of both males and females are infected (Turelli, 2010). In addition, since the infected mosquitoes persists in the population, mosquito releases of the infected mosquitoes is done once and hence its very cost effective as compared to other techniques where releases have to be done regularly inorder to maintain the population of infected mosquitoes. This method also has the advantage of eliminating the laborious process of sex-sorting prior to the release of modified or infected mosquitoes that is usually performed when implementing the sterile insect technique. This is because in *Wolbachia*-based technique both male and female infected mosquitoes are released to the wild population (Dorigatti et al., 2017).

2.12.3 Genetic modification of mosquitoes through gene drive

Mosquito populations can be genetically modified through gene drive method which involves mosquito populations being interbred and in the process desirable genes are transferred in the population though there is a fitness cost associated with the genetic procedure. This mode of genetic modification has found application in mosquito vectors of malaria and as a way of malaria control in that the transmission of malaria parasites in mosquitoes is significantly reduced and also there is reduction of fertility in the female mosquitoes and consequently the malaria vector population is minimized (Champer et al., 2016; Sinkins & Gould, 2006).

Gene drive procedures is whereby a transgene bearing desirable characteristics is passed on to a target population and within a matter of time the insect population has been modified and this is dependent on the number of the mosquitoes that are released, the cycle of reproduction of the insects targeted and also the vector density in the wild by the time of release of the modified population (Gantz et al., 2015). There are several genetic elements that can be applied in gene drives but the use of clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) has received a lot of attention (Champer et al., 2016; Gantz et al., 2015; Hammond et al., 2016). Being an endonuclease it has the ability to snip genes and thereby allowing modification of the mosquito genome and this has been observed in female mosquitoes of *Anopheles gambiae* where they have been effectively sterilized (Hammond et al., 2016). Another example of their application is in *Anopheles stephensi* where the CRISPR-Cas9 system has been used in spreading genes that have antimalarial properties and thereby conferring

malaria immunity in the mosquito (Gantz et al., 2015). Since this system is promising it could be experimented with vectors like *An. aegypti* and any other vector to prevent virus transmission (Kistler et al., 2015). Despite the system being very appealing, there could be other issues that are likely to arise for example, application of the system may lead to extinction of the vector population or may be the gene drive system could extend beyond the area of application (Esvelt et al., 2014; Webber et al., 2015).

2.13 Insecticide resistance

The World Health Organization defines insecticide resistance as a characteristic exhibited by insects whereby they have the ability to withstand lethal doses of pesticides. Continued application or use of pesticides or insectides leads to a selection pressure in the insects for the particular chemical compound being used and in the long run the insecticide becomes ineffective in the control of target organisms like mosquitoes. Once resistance has developed in insects and it becomes difficult to get insecticide formulations that can be effective on the resistant organisms (Brooke et al., 2001; Chandre et al., 1999; Diabate` et al., 2002, Yadouleton et al., 2009). The rate of reproduction in mosquitoes is high, giving rise to offsprings that have the ability of adaptation to changes that occur in the environment like pollution and also agricultural activities where there is increased use of insecticides or pesticides (Oliver & Brooke, 2018). One of the ways resistances to insecticides develop in mosquitoes is through selection pressure that takes place when there is exposure to minimal levels of pesticides over a period of time especially when spraying is not done properly and the mosquitoes have the ability to adapt to the doses and reproduce because they have developed resistance to the chemicals (Poupardin et al., 2008). Southern Benin paints a good picture of pyrethroid resistance that came about because of selection pressure that emanated from human wastes and oils pollution in the urban areas. This greatly hampers the effective use of insecticides and pesticides in the community (Djouaka et al., 2008). Behavioral plasticity and insecticides resistance have permitted mosquitoes to either resist insecticides or circumvent them through avoidance. Therefore, this limits the effectiveness of vector control tools such as long-lasting insecticides-treated nets (LLINs) or indoor residual sprays (IRS), thus leading to malaria transmission (Rydzanicz et al., 2009). In order to combat malaria transmission especially in urban settings, novel approaches will require to be applied in malaria control toolbox (Rydzanicz et al., 2009; Zhu et al., 2017).

2.14 Environmental pollution and ecology of malaria vector

The rate of urbanization is high in the third world countries with the African continent recording the highest rate of urbanization (United Nations, 2004; Zeigler et al., 2003). It is very interesting to note that at least half of the global population lives in the urban areas occupying an area of approximately 3% of the entire land area (Angotti, 1993; UNPF, 1993). In the 19th century the total number of people that were living in the urban areas was 5% but this number has risen to 47% in the 21st century and the projection is that the population in the urban areas is likely to shoot to 65% by the year 2030 (United Nations, 1990; United Nations, 1991; United Nations, 2003). However, urbanization comes with its own fair share of challenges in developing countries like Kenya because urban services in most cases do not keep up with the pace of increasing rate of urban development. The most striking features with urbanization is that there is inefficient treatment of sewages that has seen raw wastes being discharged in the environment, there is improper waste disposal from industries, hospital placements and hotels just to mention afew and in most cases wastes are thrown in open pits and dump sites (KNBS, 2017).

Industrial, agricultural and domestic activities usually release the highest amounts of substances or compounds that pollute the environment and most of those effluents find their way into rivers, streams and lakes (Biney et al., 1994). The high rate of pollution in the environment have necessitated tolerance of mosquito vectors of malaria to polluted environment and it has been observed that the vectors have undergone niche expansion to survive and reproduce in polluted environments occasioned by the human activities especially following urbanization (Awolola et al., 2007; Djouaka et al., 2007; Mireji et al., 2006). The ultimate fate of this niche expansion to polluted habitats is that there will be selection pressure in the mosquito vectors such that they will be less susceptible to the insecticides that are used for vector control approaches (Billy et al., 2012; Oliver & Brooke, 2016).). In sub-Saharan Africa, most vectors have already developed resistance to the pyrethroids that are used in treatment of mosquito bed nets and this could therefore suggest that alternative vector control strategies need to be devised (Ranson et al., 2009).

As a consequence of urbanization, malaria in the urban areas have been reported and it is a matter of great public health concern in that the urban areas can no longer be overlooked in the control of malaria and hence in the sub-Saharan Africa there is an initiative referred to as Rapid Urban Malaria Appraisal (RUMA) that aims at controlling malaria disease especially in the

urban areas (Wang et al., 2005). There is rise in pollution and creation of undesirable conditions for mosquito larval development that goes hand in hand with urbanization but even when that is the case, malaria disease transmission still occurs in the urban environments (Hay et al., 2000; Robert et al., 2003). The pointers responsible for transmissions of urban malaria are issues to do with expansion of the niche for the mosquito due to tolerance to pollution that is a product of increased industrial, agricultural and domestic activities (Awolola et al., 2007; Matthys et al., 2006; Omlin et al., 2007; Sattler et al., 2005).

Increased industrial activities have led to massive contamination of the environment globaly due to improper disposal of wastes (Awolola et al., 2007). The gateway for environmental pollution majorly arises from human activities and also from natural activities. The human factor in the production of pollutants mainly involves industrial activities that release air-borne substances polluting the air and also release of toxic compounds in form wastes that are improperly disposed. Agricultural activities that are practiced by man also aid contaminating the environment especially through the use of fertilizers to improve crop yields, herbicides and pesticides have also been extensively used to control pests and all their wastes are released in the environment. Other activities that bring about environmental pollution are activities like mining that release heavy metals to the environment, improper solid waste disposal and sewage effluents (Onyari & Wandiga, 1989). The natural ways through which the environment gets contaminated is when rocks wither releasing minerals in the environment and this happens especially in areas where a lot of volcanicity has taken place (Halim et al., 2003).

Research has been done extensively regarding the larvae habitats in the rural areas in sub-Saharan Africa that are mostly characterized with clean habitats bearing minimal pollution (Sattler et al., 2005). However, mosquito vectors have been observed to breed in diverse environments which are polluted with industrial wastes, domestic wastes and agricultural products like pesticides. This has been facilitated by the adaptation of the mosquito vector to pollutants that comes about from expression of enzymes that detoxify the toxins present in the polluted environment (Strode et al., 2006). A previous study has shown that *An. gambiae* mosquito has the capability to tolerate cadmium and lead heavy metals and it does so by the expression of cytochrome p450 genes and these genes are expressed at different levels between the males and females mosquitoes (Musasia et al., 2013). When *Aedes aegypti* was exposed to various xenobiotics and parasites of malaria, they responded through expressing different forms

of enzymes involved in detoxification of the pollutants and pathogens (Felix et al., 2010; Poupardin et al., 2008; Poupardin et al., 2010).

Heavy metals have the capacity to persist in the environment for prolonged periods of time posing health related issues to both animals and humans (Gisbert et al., 2003; Halim et al., 2003). Zinc, Copper and Cadmium heavy metals are common environmental contaminants in urban and industrial settings (Mireji et al., 2008). Zinc, copper and iron are required in trace levels for cellular and biological processes being crucial components in enzymatic reactions. However, cadmium has no biological role in living organisms (van Straalen & Roelofs, 2005) and it has a half life of approximately 18 years (Forstner, 1995). Cadmium heavy metal is more toxic in comparison to other heavy metals and its exposure to insects has many adverse side effects which involve effects on the signal transduction pathway and also effects that relate to oxidative stress in the insects (van Straalen & Roelofs, 2005).

To determine the heavy metals composition in Africa, most studies have focused on analysis of the sediments from dams, rivers, lakes and oceans (Biney et al., 1994). In Kenya, focus has been on the sediments of Lake Victoria (Onyari & Wandiga, 1989; Urasa & Onyari, 1986; Wandiga & Onyari, 1987). Analysis of heavy metals at the Kenyan coast has also been undertaken (Rees et al., 1996; Kamau, 2002). In the Rift valley region analysis of heavy metal composition has been done in Lakes Nakuru, Elementaita, Naivasha, Baringo and Bogoria (Ochieng' et al., 2007). In most the studies carried out, researchers have found out that heavy metals are present in the sediments analysed and in some cases there are heavy metals that have been reported whose levels are higher than expected mainly being attributed to industrial and agricultural activities (Ochieng' et al., 2007; Onyari & Wandiga, 1989).

2.15 Heavy metal pollution in Kenya

Metals are termed as heavy when their specific density is about of $5 g/cm^3$ (Ferguson, 1980). There are metallic elements like iron (Fe), copper (Cu) and Zinc (Zn) among others that have important biological functions in plant and animal life by being crucial components in enzymatic reactions (Bondy, 2016). However, heavy metals such as cadmium (Cd), mercury (Hg), nickel (Ni) and tallium (TI) among others, although useful in industrial and agricultural technology, can be potentially hazardous in combined or elemental forms to living organisms (Southon et al., 2013; van Straalen & Roelofs, 2005).

Heavy metals have a high solubility in water and the metals that raise a lot of concern when present in the environment are mainly Pb, Cr, As, Cd, Cu and Zn (Martin & Coughtrey, 1982). Cadmium toxicity is felt even when low concentrations are present in the environment because it leads to detrimental effects such as mutations in organisms that are in contact with this heavy metal (Emre et al., 2013). The solubility of heavy metals in water makes their concentration to be high in water bodies and also in aquatic organisms as they are readily taken up. The major sources of heavy metal pollution are industrial processes that deal directly or indirectly with heavy metals, agricultural activities are also responsible for high doses of heavy metals mainly emanating from the use of insecticides, pesticides and herbicides that have heavy metal components in them. Also the process of urbanization has compounded the issue of heavy metal pollution and all the wastes find their way to water bodies and thereby leading to detrimental effects on the environment and the aquatic organisms (Kinuthia et al., 2020; Qiao-qiao et al., 2007). A study on the population status of Masinga reservoir in Kenya whose catchment comprises of three river subsystems, namely Tana River, Thiba River and Thika River indicated the presence of heavy metals (Nzeve et al., 2015). The main sources of heavy metal pollution being agro-based industries, agro-chemicals, urban effluent, car washing, soil erosion, river bank encroachment and industry discharge (UN-Water, 2006).

2.16 Methods used in differential gene expression

2.16.1 Gene fishing technique

Gene fishing is a technique used for the identification of genes that are differentially expressed in RNA or DNA samples that have been subjected to a particular treatment. This is enhanced by the application of the Annealing Control Primer (ACP) technology which is a sensitive technique applied in the amplification of nucleic acids and it ensures that only specific products are amplified (Hwang et al., 2004). The advantages attached to this technology is that only a small amount of sample is required and also the detection of the nucleic acid products is cheap since it is normally done on agarose gels that have been stained with ethidium bromide.

The Gene Fishing Technology procedure involves reverse transcription step that is used for the synthesis of the first strand of complementary DNA from total RNA samples extracted from the organisms of interest by the application of dT-ACP1. The 3’-end of dTACP1 bears a series of polyT sequences that form complexes with the complementary polyA tail on the mRNA

strand. The hybridization leads to the formation of the first strand of the complementary DNA whose 5' end carries the sequence of dT-ACP1. An arbitrary ACP and dT-ACP2 components are placed in a PCR tube and then the cDNA which had been obtained in the previous step is diluted and added into the same tube. The structure of the arbitrary ACP is that the 3'-end core portion which has about ten nucleotides is complementary to a portion of the first strand of the cDNA. At this point the first PCR reaction is carried out under stringent conditions allowing annealing to take place specifically between the 3' end of the arbitrary ACP and the diluted cDNA and no annealing takes place between the cDNA and dT-ACP2. A second stage polymerase chain reaction is carried out on the components in the PCR tubes under high stringency ensuring that annealing only happens between dT-ACP2 and the arbitrary ACP. These stringent annealing and amplification procedures ensure that only the PCR products of interest are amplified. The gene fishing technology has found application in different organisms facing various health issues and also in organisms at different stages of growth and development (Cui et al., 2005; Hwang et al., 2005).

2.16.2 Subtractive hybridization

This technique has found application in studies that deal with determination of genes that are differentially expressed in particular cells or tissues after a particular treatment or genes expressed in specific stages in the development of organisms like animals, plants, insects and bacteria among others. When organisms are subjected to different treatments or raised in different environment other than the ones they are normally found in, they respond by alteration of the patterns of gene expression thereby eliciting the production of proteins that will enhance their survival in the new conditions of treatment (Lin et al., 2007). This technique therefore helps in the comparison of two mRNA samples that are obtained from a treated population and from an untreated population and therefore it is possible to deduce genes expressed in one population (treated) and not in the other (control) (Diatchenko et al., 1996). Through subtractive hybridization technique many genes that are functional in different states in cells and tissues have been identified (Chang et al., 1989; Kunkel et al., 1985; Lamar & Palmer, 1984; Littman et al., 1985; Maddon et al., 1985 Nassbaum et al., 1987). After subtractive hybridization technique, there are two scenerios that mostly arise and the first case is where the differential DNA fragments of interest are of high quality and quantity and in such cases isolation and detection is

straight forward but in the cases where the DNA quantity is low, PCR procedure is usually undertaken to amplify the DNA so as to enhance other down stream processes (Cecchini et al., 1993; Wang & Brown, 1991; Wieland et al., 1990). The procedure for this technique entails the extraction of total RNA from both populations under study and then mRNA is prepared for the two populations. The messanger RNAs through the process of reverse transcription form complementary DNAs for both populations and they are referred to as the tester cDNA and the driver cDNA. Hybridization is then allowed between the driver and the tester cDNAs. The draw backs to this technique is that many hybridization reactions must be carried out for this technique to be successful and also the fact that rare transcripts cannot be obtained through this technique (Davis et al., 1984; Duguid & Dinauer, 1990; Hara et al., 1991; Hedrick et al., 1984; Sargent & Dawid, 1983).

2.16.3 Bulk Segregant Analysis (BSA)

This technique is used in genomic analysis for the identification of particular markers of interest (Michelmore et al., 1991). The procedure for this technique is that crosses are made between segregating populations and then DNA is obtained from the two bulk populations is compared. When two pools contrast for a particular trait, analysis is carried out to determine the distinguishing markers between the individuals under study. The assumption made in this form of analysis is that the only region that the two bulked DNA samples differ genetically is the region of interest while the other regions are considered similar (Becker et al., 2011). Differences in the bulked populations are identified using molecular techniques like Restriction Fragment Length Polymorphisms (RFLP) (Dawson, 1990) or Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995).

Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique is a strong and repeatable DNA fingerprinting process (Vos et al., 1995). The effectiveness of AFLP is focused on the molecular genetic differences that exist among closely related organisms. The AFLP technology takes advantage of these variations in DNA sequence to produce “fingerprints” of specific genotypes on a regular basis. Genomic DNA is usually digested with a common and unusual site-cutter (Masiga et al., 2000; Vos et al., 1995). These DNA fragments are ligated with ‘adapter' oligonucleotide sequences, which are made up

of two parts: a core sequence and an enzyme-specific sequence. The primers are made up of three parts and are used in a PCR reaction with high-temperature annealing. These primers are made up of an enzyme-specific sequence specific to adapter-sequences, a selective extension on the 3' end of the primer that determines the adapter's specificity to the enzyme by one to three arbitrary nucleotides and the primer's core sequence (Bachem et al., 1996). Autoradiography or non-radioactive methods are used to visualize polymorphisms on polyacrylamide gels.

The presence or absence of restriction fragments, rather than length variations, is shown by amplified fragment-length polymorphism (AFLP) markers, which are the most common. When compared to other marker technologies such as randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and microsatellites, the advantages of AFLP are that it not only has a higher reproducibility, resolution and sensitivity at the whole genome level, but it also has the ability to amplify multiple fragments at once (Mueller & Wolfenbarger, 1999). Furthermore, AFLP needs no prior sequence knowledge for amplification, making it extremely useful in the analysis of taxa such as bacteria, fungi and plants, where much about the genomic makeup of different species is still unknown (Meudt & Clarke, 2007).

Restriction Fragment Length Polymorphism (RFLP)

RFLP is a technique for distinguishing species based on patterns produced by DNA cleavage. When DNA is digested with a restriction enzyme, the length of the fragments formed will differ if the distance between sites of cleavage of a specific restriction endonuclease differs between two species. Restriction endonucleases are enzymes that, depending on the enzyme, cleave DNA molecules at various nucleotide sequences.The length of enzyme recognition sites is normally 4 to 6 base pairs. The number of fragments produced is usually proportional to the length of the recognition sequence. If the nucleotide sequences of two molecules differ then fragments of various sizes will result. Using gel electrophoresis, the fragments can be isolated. Bacteria genera have been the source of restriction enzymes and these enzymes are thought to play a role in the cell's protection against invading bacteria viruses (Narayanan, 1989). Polymorphisms are hereditary anomalies that can be observed in more than 1% of the general population. Restriction Fragment Length Polymorphism can be used in many different settings to accomplish different objectives which include: They can be used for forensic applications such as in paternity cases or criminal cases to determine the source of a DNA sample, RFLPs can be

used determine the disease status of an individual for example it can be used in the detection of mutations especially known mutations and they can be used to measure recombination rates which can lead to a genetic map with the distance between RFLP loci being measured (Dawson, 1990). Some restriction enzymes, such as Hind II from Hemophilus influenza, cleave at the center of their recognition sites, resulting in fragments that do not bind together, whereas others, such as EcoRI from E. coli, cleave away from the center of their recognition sites, resulting in fragments with sticky ends (Narayanan, 1991). Restriction enzymes are used in a number of different applications whereby the major benefit is that they digest DNA into manageable fragments that can be cloned or subjected to various types of electrophoresis.

2.16.4 Microarrays

Microarrays are used in molecular Biology to determine expression profiles of genes and proteins (Schena et al., 1995). Microarrays initially found application in the genome analysis of *S. cerevisiae* (Lashkari et al., 1997). There have been improvements regarding the probes that are currently used in that previously DNA probes were normally obtained from a library of prepared clones but presently chemical oligonucleotides have become the probes of choice. Large numbers of parallel analysis of either genes or proteins have been undertaken using very little amounts of starting material (Sandoval et al., 2011). In addition to the high throughput of this technology the quality of the data obtained has also increased tremendously with improved labeling criteria and mode of production of the arrays (Karakach et al., 2010; Leung & Cavalieri, 2003; McCall et al., 2011; Priness et al., 2007; Thirwell et al., 2010). The microarray has a solid support material that comprise of glass material whose surfaces bear aldehyde groups where probes are usually immobilized. The modes of immobilization of the probes are by either the use of spotters that are able to print the probes on the glass surface or alternatively by automated way of synthesizing the probes. This is usually followed by the blocking procedure using non-reactive substances and this is meant to prevent non-specific binding between the probes and the targets. In most cases microarrays are easy to handle since their size is like that of a microscopic slide and this enhances the processing of several samples in a given assay and thereby producing large amounts of data (Howbrook et al., 2003; Karakach et al., 2010).

Microarray data is usually obtained by application of various strategies and biomolecules but in all cases the procedures are similar. The principle behind this technique is that there is

hybridization between the probes that are usually immobilized on a glass surface and the labeled target molecule (Wheelan et al., 2008). For a microarray study to be undertaken, DNA or RNA which are the most commonly used targets have to be extracted using the optimized extraction methods, the nucleic acids are then bound onto silica membranes and later the DNA or RNA are eluted using buffers with a lower salt concentration and this is followed by the labeling step. In other cases labeling is often done with at the enzymatic stage where there is incorporation of labeled nucleotides into the targets (Schaferling & Nagl, 2006). The fluorescent dyes that have found application in microarrays are mainly Cy3 and Cy5 (Liang et al., 2003). The labeled fragments are usually purified and then a buffer that has components for facilitation of the hybridization process are mixed together and applied to microarrays and an overnight incubation is carried out to enhance the hybridization process. After overnight incubation the molecules that remain unbound are usually washed away and then detection with dyes of specific wavelengths is carried out. The intensities of signals demonstated are dependent on the number of target molecules that bind to the probes. Bioinformatics analysis is applied to analyse the microarray data obtained (Pulverer et al., 2012). The advantages of microarray technology are that from a single sample very many probes can be detected simultaneously and therefore this technology has found application in genomic analysis of variations in different organisms and analysis of single nucleotide polymorphisms (SNP) among others (Bier et al., 2008; Khelurkar et al., 2017). In addition, the technology is cheap and only a small amount of sample is needed for the reactions. Microarrays have also found application in the analysis of expression profiles generated from mRNA. For example, mRNA can be extracted from a healthy tissue and also from a diseased tissue then a complementary DNA is formed through the process of reverse transcription using mRNA as the template. The two cDNA molecules from the healthy and the diseased tissues are then labeled using the two commonly used fluorescent dyes which are Cy3 and Cy5. This is then followed by pooling the labeled cDNA and then hybridization onto the arrays and later scanning of the microarrays takes place and the images can be analyzed (Duggan et al., 1999; Schena et al., 1995).

2.16.5 RNA sequencing

Techniques such as northern blotting and quantitative polymerase chain reaction (qPCR) were previously relied upon for the study of gene expression but these are low-throughput

techniques especially in the determination of transcripts. Currently, more versatile techniques like transcriptomics have come up that are effective for carrying out gene expression studies (Kukurba & Montgomery, 2015). Microarray based studies that rely on hybridization between the targets and the DNA probes were the first of a kind to undertake transcriptomics related studies bearing the advantages of being cheap and high throughput technology (Schena et al., 1995). At the same time, there are draw backs that are experienced with the microarray technology and these mainly relate to the fact that the researcher that desires to use microarray must have previous knowledge regarding the sequences being studied, again when dealing with sequences that are very similar, there is a likelihood of development of artifacts during the hybridization process. Another draw back is that quantification accuracy problems usually arise when genes being interrogated are either expressed at very low levels or at very high levels (Casneuf et al., 2007; Shendure, 2008). Due to the limitations that are experienced with microarray technology and other hybridization approaches, there has been development of other techniques such as RNA sequencing where transcriptome analysis is usually undertaken through the determination of the sequences of the transcripts. The term 'transcriptome' can be defined as the sum total of all the transcripts present in a cell at a particular physiological state or stage of development in an organism. Transcriptomics studies are necessary for the elucidation of transcripts expressed in cells during particular physiological processess like growth and development of diseases among other conditions under study (Cloonan et al., 2008).

RNA-sequencing (RNA-seq) is a sequenced-based approach whose procedure involves extraction of RNA from the organism or sample of interest and the RNA is used as the template to prepare complementary DNA. This is then followed by construction of cDNA libraries which are later sequenced on a platform of next generation sequencing (NGS) (Han et al., 2015; Marguerat et al., 2008). This advanced technology of determining expression of genes has circumvented the limitations that go hand in hand with technologies like the use of microarrays and Sanger sequencing methods (Shendure, 2008). The advantages of RNA sequencing are that it gives a better understanding of the organisms entire transcriptome and as such the positions of the exons and the introns and the boundary between them can be identified and therefore it becomes even easier to point out the transcription start sites (Morin et al., 2008; Nagalakshmi et al., 2008; Tsuchihara et al., 2009; Wilhelm et al., 2008). Through this quantitative technology, accurate determination of the expression levels of RNA is obtained as compared to the

hybridization techniques like microarrays (Mortazavi et al., 2008). RNA sequencing is a powerful technique such that the quantification of all transcripts in a cell can be determined accurately as compared to other gene expression techniques (Han et al., 2015). However, the draw backs associated with this technique are that it is expensive and a lot of Bioinformatics knowledge is required for the analysis of data and also challenges that relate to library construction (Wang et al., 2009).

2.17 Proteomics

2.17.1 History of proteomics

Proteomics first came about around 1955 and it refers to the characterization and analysis of the complete set of proteins that are present in a cell, tissue or organism (Persidis, 1998; Wasinger et al., 1995; Wilkins et al., 1996). Proteins that are contained either in cells or tissues of an organism differ in their abundance and also different physiological activities or occurences in their systems or environments trigger expression of particular proteins in response to the stimulus that they are subjected to (Wang et al., 2008a). The physiological triggers could include growth, development, disease condition, pollution in the environment among other activities and therefore proteomic studies are used to determine not only the proteins present in a particular stage or state in an organism but also to identify the expression patterns of proteins after a certain physiological event or events (Wang et al., 2008a). Proteins are very important in the life of organisms because they are involved in diverse functions like cell-cell communication, immune response, cell motility, signal transduction, enzyme catalytic activity, mitosis and transport among other processes (Fields, 2001). The proteomics technology has advanced research based on proteins and studies are undertaken to determine the expression pattern of proteins in a diseased tissue, the structure and functions of proteins can be elucidated and the interactions between proteins can be interrogated with ease (Graves & Haystead, 2002; Fields, 2001). It is important to understand that the genome does not change with changes in the environment or physiological changes that take place in or around an organism but the proteome is ever changing in response to the environment or physiological changes like growth and development, disease development or tolerance to xenobiotics like heavy metals. Proteins involved in particular events in an organism can be determined by comparison of two different cell states such as treated versus untreated or control and healthy versus diseased cells. Proteomic studies

are carried out to determine proteins that are differentially expressed, in terms of up-regulation or down-regulation after subjection of an organism to particular treatments (Kim et al., 2004b). When the analysis of the genome is compared to that of the proteome it can be concluded that proteome analysis is more detailed and generates huge data sets as compared to the analysis of the genome The proteomics procedure mainly entails the extraction process of proteins followed by the separation of the proteins then the proteins are quantified and lastly a suite of Bioinformatics tools are used for data analysis (Claassen et al., 2012; Nesvizhskii & Aebersold, 2005). Separation of proteins and peptides is usually carried out by application of either electrophoresis technique or chromatography. The chromatographic separation techniques that have been commonly used in the separation of proteins are mainly gel filtration, affinity and ion exchange chromatography. Even though these chromatographic techniques are selective, most researchers prefer to use electrophoretic procedures for protein separation in the form of either one dimensional polyacrylamide gel electrophoresis or two dimensional polyacrylamide gel electrophoresis (PAGE) (Penque, 2009). The techniques used in the separation of proteins are usually combined with mass spectrometry (MS) and these are powerful tools that are applied in proteomic studies (Kolch et al., 2005). Mass spectrometry is a high throughput technology that is specific, sensitive and its detection range is appealing and therefore it has been used to identify and quantify proteins (Aebersold & Mann, 2003; Foster et al., 2006; Washburn et al., 2001). Initially, mass spectrometry was not considered as a method of choice in protein studies and this was dictated by the fact that proteins have a large size and they are also fragile and therefore they would not undergo the ionization processes. However, the development of soft ionization techniques that are able to generate ions from large molecules without significant fragmentation have aided in overcoming these challenges and therefore enhanced a detailed study of proteins (Chaurand et al., 1999; Koomen et al., 2005). These soft ionization techniques are electrospray ionization mass spectrometry (ESI) and matrix assisted laser desorption ionization mass spectrometry (MALDI). The two main ways of identying proteins in MS are bottom-up and top-down approaches. ‘Bottom-up’ strategies involve the digestion of proteins and then peptide detection and they are the most common for large-scale analysis of complex biological samples. Methods like peptide mass fingerprinting (PMF) or further peptide fragmentation are used to identify proteins. Intact proteins are studied by mass spectrometry methods in ‘top-down’ approaches (Aebersold & Mann, 2003).

Application of mass spectrometry technology that is fast and sensitive has enhanced proteins to be characterized and identified at a faster rate. This has been facilitated by the use of a mass spectrometer which has the ability to determine the molecular weight of a particular compound. This is possible because in the mass spectrometer ionization process takes place and there is induction of either a positive or a negative charge then the ions are separated according to their mass and the mass to charge ratio of the ions is later determined (Chaurand et al., 1999; Koomen et al., 2005).

Proteomics sample preparation

Proteins are vital to organisms' survival because they are involved in a variety of processes such as cell-to-cell contact, immune response, cell motility, signal transduction, enzyme catalytic activity, mitosis and transport among many others (Fields, 2001). There are various methods that are used for the extraction of proteins. The methods involves the use kits that have cost implications and also reagents like trizol which are cheaper compared to the optimized kits for protein extraction. The protein yield differs depending on the extraction method used and therefore optimization processes have to be undertaken inorder to maximize the protein yield. Optimization reactions to improve the protein yields mainly puts into consideration factors like the sample source which could be from a whole organism, tissue, fluids like serum or plasma whereby the right sample amount has to be used. Also the levels of expression of the proteins and their location and also the strategies to be used for protein analysis have to be put into consideration (Bodzon-Kulakowska et al., 2007). The proteins have to be denatured and this procedure transforms proteins from the three dimensional structure to a linear structure that enhances analysis of the proteins. A lysis buffer comprising of chaotropic agents and detergents is usually used to enhance the denaturation and solubilization of proteins (Zhang et al., 2013). Dithiothreitol (DTT) which is a reducing agent is used to treat the linearized proteins to prevent the formation of disulfide bonds and the solution is then alkylated using iodoacetamide (IAA) that reacts with the free sulfhydryl groups from cysteine residues and hence work to prevent further formation of the disulphide bonds in the proteins. This is then followed by overnight digestion of proteins using proteases with trypsin being the protease that is commonly used in most studies (Switzar et al., 2013).

2.17.2 Separation of proteins and peptides

Extracted proteins are available in different concentrations and also in complex forms and therefore various methods are available for the separation of the proteins and a researcher has to choose the right separation technique for a successful proteomics study (Aebersold & Mann, 2016; Smith & Kelleher, 2013). There are two main separation techniques that are used in proteomics research and these are gel-based and chromatography-based separation techniques.

One-Dimensional electrophoresis

One-Dimensional electrophoresis comprises of isoelectric focusing that is carried out in the first dimension of a 2-dimensional electrophoresis. Separation through the isoelectric focusing technique is where separation of the proteins is based on their isoelectric points. The isoelectric point of protein is the pH at which a protein has equal number of negatively charged ions and positively charged ion giving a protein a net charge of zero. Separation is by enhanced by carrier ampholytes that are used to form a pH gradient (Klose, 1975; O`Farrell, 1975). The draw backs associated with isoelectric focusing technique are minimal reproducibility, low resolution and challenges arise when separating proteins with extreme pH conditions (Weiss & Gorg, 2009). The carrier ampholytes offer a pH gradient for the separation of proteins and they bear the characteristics of high buffer capacity, their molecular weight is low and they have a good range of pKa values that extend from pH 2-12 and they mainly comprises weakily acidic and weakily alkaline mixtures. When the set up for separation of proteins is completed, it is then connected to a power supply and the ampholytes migrate based on their charges to the positive or negative electrodes. Proteins possess a negative charge when the pKa values are above the pH and they migrate towards the anode and vice versa. Migration therefore takes place until the point where the pH and the pKa are equal and at that point the net charge is zero and no further migration takes place. There were draw backs like batch differences that were associated with the use of carrier ampholytes in isoelectric focusing technique and this prompted the development of immobilised pH gradients and they have since been modified to increase their efficiency (Bjellqvist et al., 1982; Gorg et al., 1988). The immobilization procedure is achieved by the use an acrylamide matrix where covalent binding of the pH gradient is done when casting and this process leads to the formation of a pH gradient that is more stable than the gradient formed by the carier ampholytes. This modified gradient ensures that IEF takes place in a more

steady state and thus becomes very reproducible and the resolution of this technology has greatly improved (Gorg et al., 1988; Gorg et al., 1991; Righetti & Bossi, 1997). The modified pH gradients have a broad pH range of 2.5-12 and this range allows efficient separation of the proteins (Gorg et al., 2004). The immobilised pH gradients have found application in separation of proteins by isoelectric focusing for use in proteomic studies (Gorg et al., 2009).

2-Dimensional gel electrophoresis

The principle behind the 2-dimensional gel electrophoresis technique is that protein separation takes place by the first dimension using isoelectric focusing that utilises isoelectric points for separation. The second dimension of separation is usually by size which is done perpendicular to the initial dimension and this makes use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The role of the anionic detergent referred to as SDS is to disrupt hydrophobic interactions between the proteins that are meant to stabilize the proteins and the anionic nature of SDS masks the native charges in the proteins such that all the proteins in the sample are negatively charged and this serves to ensure that separation of the proteins in the second dimension is purely based on their size. Thiols like 2-mercaptoethanol are also used together with SDS and they play a major role of disruption of the disulphide bonds present in the proteins thus unfolding the protein structure. The gel matrix for the separation of proteins is offered by acrylamide and N, N'-methylenebisacrylamide which is cross linking reagent and therefore forms a meshwork of different pore sizes since separation is based on the molecular weights of the proteins (Raymond & Weintraub, 1959). SDS-PAGE is carried out in a vertical system that allows many parallel runs to be done and it is very efficient especially when undertaking analysis in large scale (Anderson & Anderson, 1996). When running this technique a discontinuous buffer system is usually used (Laemmli, 1970) and different concentrations of acrylamide solutions generate different pore sizes and therefore the right concentration must be prepared to ensure efficient separation of the proteins in the polyacrylamide gel (Zimmy-Arndt et al., 2009).

2.17.3 Chromatography-based separation methods

Separation of proteins based on chromatographic techniques have also found application in proteomic research where techniques like affinity chromatography and size exclusion

chromatography have been applied in the first dimension (Wolters et al., 2001) and in the second dimension reversed phase liquid chromatography and is usually combined with mass spectrometry.

Ion exchange chromatography

Proteins are composed of different types of amino acids and they bear both positive and negative charges and therefore these properties have been exploited to enhance the separation of proteins based on charged ions (Gupta et al., 1983; Kopaciewicz & Regnier, 1983). For proteomic analysis, ion exchange chromatography has been applied for separations of peptides as opposed to crude protein separations and this has been done in conjuction with high performance liquid chromatography. Strong cation exchange (SCX) which is a form of ion exchange chromatography has been exploited for the separation of peptides in the first dimension based on charge differences (Washburn et al., 2001; Weston & Brown, 1997; Zhou et al., 2007). The mode of action is through electrostatic attractions whereby ions of like charges are able to exchange with the analytes for binding to the matrix (McMaster, 2007). Ion exchange chromatography has two exchangers and these are a cation exchanger that exchanges positively charged peptides with a compound of similar charge attached on a negatively charged matrix and anion exchanger on the other hand exchanges negatively charged peptides with a compound that is also negatively charged attached to a positively charged matrix. In both cases of cation and anion exchangers there are both weak and strong forms of the ion exchangers (McMaster, 2007). After the cationic or anionic peptides have been bound, the next step is eluting the peptides and this is usually achieved by the application of solutions with varying ionic strength for the peptides to migrate (Nakamura et al., 2008). Other elution modes include varying the pH of mobile phase (Dai et al., 2005; Manadas et al., 2009), or increasing the concentration of the ions that were displaced and these ions will again exchange with the peptides through competitive mode of action. Strong cation exchanger has found application in most proteomics studies whereby during elution process the peptides bearing the least net positive charge is eluted first (Delmotte et al., 2007; Essader et al., 2005; Gilar et al., 2005a).

Size-exclusion chromatography

Two-dimensional liquid chromatography has incorporated size-exclusion chromatography (SEC) as the first dimension in the separation of proteins and it exploits the differences in sizes of the proteins. This separation technique has been sidelined in proteomics yet it is stable and is faster in the analysis of samples (Lecchi & Abramson, 1998; Lecchi & Abramson, 1999). There have been proteomic studies that have used this technique in conjuction with reversed phase high performance liquid chromatography for the separation of peptides (Opiteck et al., 1997; Opiteck et al., 1998a; Opiteck et al., 1998b). In most of the proteomic studies, the size exclusion chromatography is connected with the soft ionization technique like electro spray ionization with mass spectrometry to assist in the identification of peptides. There are studies that have effectively applied this technology in proteomics studies (Gao et al., 2003; Liu et al., 2002; Nemeth-Cawley et al., 2003; Whitelegge et al., 2002).

Immobilised Metal-Ion Affinity Chromatography (IMAC)

This form of affinity chromatography is a separation technique that depends on the association between analytes and metallic ions that have been immobilised on a matrix. The matrix which acts as a solid support contains functional groups that make interactions with the metallic ions leading to their immobilisation. This technology is mostly applicable to the separation of phosphorylated proteins and peptides because their affinity to the metal ions is high and they end up forming complexes that are stable. The metal ions that are commonly used in this separation technique include zinc, chromium, ferric iron, aluminium, nickel and copper. The multidenante ligand is usually immobilised onto the stationary phase and the ligand is used to chelate the metallic ions. Proteins which are digested by proteases like trypsin are usually directed to the affinity column that contains the chelated metal ions and binding take place. They are later eluted in a selective way into a column of reversed phase and then separation is undertaken using the nano separation column (Carrascal et al., 2008; Chien et al., 2011; Engholm-Keller et al., 2011; Gan et al., 2008; Gonza`lez-Ortega et al., 2012; Novotna et al., 2010; Wang & Li, 2010; Zhou et al., 2013). When titanium dioxide is used in this technology, binding to the column occurs for the phosphopeptides and also for non-phosphopeptides that are acidic in nature and this complicates the affinity technique (Ficarro et al., 2002). After binding of the phosphopeptides, elution usually follows and this is done through the use of reversed phase

trap column and the whole technology is online and automated. The elution modes that are applicable in IMAC is displacement of compounds through competitive elution and also changing the pH levels of the mobile phases and those changes lead to elution of the phosphopeptides in columns for further downstream processes that will identify and quantify the phosphoproteins. This technology has found application in proteomics studies for separation of the peptides since there are minimal cost implications involved especially in the purchase of the stationary phase. The association between the metal ions and the peptides is very high, specific and the binding level is high. Therefore this technique has been applied in the isolation of peptides and viral vectors (Jiang et al., 2004; Jiang et al., 2006; Kenig et al., 2006; Riggs et al., 2001; Vijayalakshmi, 1996; Ye et al., 2004; Zachariou, 2004).

Reversed-phase chromatography

Reversed phase chromatography is a form of High performance liquid chromatography that utilizes a stationary phase which is non-polar and a polar mobile phase that uses mostly water. The stationary phase is usually made of silica support that contains modified long chain hydrocarbons. The principle of separation using this technique is that samples make interactions between the stationary phase and the mobile phases whereby components of the sample that bind strongly to the stationary phase move slowly through the column as compared to the those that bind weakily (Dong, 2006). The samples that bind weakily on the stationary phase travel faster with the mobile phase and are eluted first and in essence separation takes place. The mode of separation is the gradient technique whereby to start with the mobile phase has high water content and then the organic component of the mobile phase is raised in a stepwise way and this enhances the separation of compounds present in a sample. The advantages that are observed because of varying the composition of the mobile phase are that the separation of components is better since the resolution is improved and the peak broadening effect is lowered. Reversed phase chromatography has found application in the separation of peptides awaiting analysis in mass spectrometry platforms. In the separation procedure of peptides, the polar peptides usually elute first because they make interactions with the polar mobile phase while the non-polar peptides are retarded in the stationary phase and as such they are eluted last (Dong, 2006). The separation of peptides has been undertaken using the reversed phase liquid chromography because this technique is very compatible with compounds that are aqueous in nature, it works

well with compounds that can be eluted in a stepwise method and this therefore enhances the separation of substances through the application of varying pH and also varying the ionic concentration of the eluting sustances (McCalley, 2010). Inorder to achieve better chromatographic peaks with this technique there are various points to consider which mainly revolve around the length of the column to be used in separation, the working temperature that has been applied to enhance efficient separation of the compounds, the stationary phase composition should be stable to enhance proper separation and also the mobile phase used should allow the stepwise change in the composition thus ensuring that gradient elution can be undertaken (Gilar et al., 2005a). For this chromatographic technique to be effective in separation and quantification of substances, the reagent that is present on the silica support ensures that the charged peptides are maintained as neutral compounds and this minimizes the repulsive effect between the peptides and the silica solid support and therefore the retention is improved and this ultimately leads to better peaks (Gilar et al., 2005b; Mccalley, 2005). Reversed-phase chromatography has found application for separation of peptides in the first dimension and this is because of its efficiency and the fact that it accommodates varying pH levels of the mobile phase (Gilar et al., 2005a). Varying the pH levels coupled with the charged nature of peptides and the stability of the stationary phase makes this technique selective in that the first dimension of separation can utilise high pH level while the second dimension of separation can be done at a lower pH level (Gilar et al., 2005a; Gilar et al., 2005b; Toll et al., 2005). The availability of stationary phases that are made of silica that has the capacity to withstand high pH levels has allowed the application of the reversed phase chromatography for the separation of peptides in the first dimension and the high pH also leads broader elution capacity (Lasaosa et al., 2009; Manadas et al., 2009; Murphy et al., 2008).

One-Dimensional and Two-Dimensional Liquid Chromatography

Liquid chromatography (LC) is a chromatographic technique for the separation of substances prior to other down stream process like mass spectrometry. The principle behind this separation technique is that the stationary phase is usually packed into a column and a solution of mobile phase is available and therefore when a sample is introduced into the chromatographic set up, it interacts with stationary phase and the mobile phase selectively (Scott, 1992). The components in the sample interact differently with the stationary phase and the mobile phase and

the substances that bind strongly with the stationary phase are retarded and they move slowly compared to the compounds that have weak or no attraction to the stationary phase. For the substances that have better interactions with the mobile phase they move faster within the column and they are normally eluted first. Therefore, different compounds have different retention times as they move through the column and this process of selective retardation through the column ensures the separation of the sample under study into its different components. High Performance Liquid Chromatography (HPLC) involves the use of high pressure to force the samples through a column with the stationary phase. The column is long and coiled to enhance better separations of the samples. One-dimensional liquid chromatography (1D-LC) is based on the application of reversed-phase liquid chromatography. This form of chromatoghraphy entails the use of stationary phase that is non-polar mainly consisting of alkyl chain like C18 that are hydrophobic in nature and a polar mobile phase mainly consisting of water combined with acetonitrile which is an organic solvent that enhances gradient elution for efficient separation of substances using this technique. This technique is easy and cheap because the instruments required are fewer as compared to the other orthogonal techniques (Davis, 1991; Dong, 1992). When separation is being done awaiting down stream process such as mass spectrometry analysis, the first dimension of chromatographic technique is usually done in conjunction with orthogonal separation techniques that either comprises of two dimensional liquid chromatography or multidimensional liquid chromatography (MD-LC). The advantages of two dimensional LC are that a small amount of sample is needed and automation is also possible with this technique. Further more this technique can be combined with mass spectrometry in an online mode through ESI (Nagele et al., 2003; Washburn et al., 2001) or offline mode through matrix-assisted laser desorption ionization (MALDI) (Machtejavas et al., 2004).

2.17.4 Visualization of proteins

Visualization of the proteins is the step that comes after proteins have been separated through gel electrophoresis. The basis of proteomics study is to determine the expression level of the various proteins expressed in response to a particular treatment, disease condition or any other stimuli and therefore visualization techniques aid in observing the different proteins in a gel. There are characteristics that any visualization technique is supposed to possess and these include the technique must be sensitive and this means that it has the capacity to identify proteins

even those with a low concentration and the technique must be compatible with all downstream processes without compromising the results. However, no single staining methodology has the ability to meet all the requirements (Gorg et al., 2004). The staining methods that are available for molecular biology research are silver staining, reverse stain, fluorescent stain, coomassie brilliant blue stain among others (Patton, 2002). The staining dye that has been commonly used for staining of proteins especially after two dimensional electrophoresis is coomassie brilliant blue. This dye has found a lot application especially in proteomics platforms because it is easy to use, cheap and it is also compatible with the methods that are incorporated in the analysis of protein after the visualization step. The draw back of this technique is that it is less sensitive and therefore it is not applicable for staining less abundant proteins (Weiss & Gorg et al., 2009; Wu et al., 2005).

Silver staining is another visualization method for proteins that have been separated using two-dimensional electrophoresis. This staining method is more sensitive and it can effectively stain low abundant proteins but its dynamic range of staining is lower than that of coomassie brilliant blue staining method. Silver staining method is more demanding in that it involves several steps in its procedure and the reaction must be stopped as per the protocol and this technique also suffers the problem of poor reproducibility between gels (Wu et al., 2005). The silver stains that are found to be very sensitive are not compatible with mass spectrometry analysis. There has been development of other silver staining procedures that have been used to stain proteins that are to be processed using mass spectrometry analysis though these modified protocols are less sensitive (Patton, 2002; Shevchenko et al., 1996; Wu et al., 2005).

Fluorescence staining is also another staining technique for proteins and it bears the characteristics of being easy to perform, sensitive and the dynamic range of visualization is broad. However, the draw backs of this visualization technique are the cost implications associated with it and this is due to the fact that the chemicals and the instrumentation that are used for the staining procedure are costly. For fluorescence staining, fluorescent dyes are used and these dyes have been in use for staining of proteins separated by SDS-PAGE and for protein blotting strategies (Wu et al., 2005). This form of staining compares well with other staining techniques like silver stain and coomassie brilliant blue staining methods in that it is sensitive and has a good dynamic range of visualization. The staining procedure can even be performed overnight without problems of over-development (Ahnert et al., 2004; Patton, 2002).

2.17.5 Mass spectrometry ionization methods

The ionization methods that are applicable in mass spectrometric methods for production of ions are mainly matrix assisted laser desorption ionization (MALDI) (Karas & Hillenkamp, 1988) and electro spray ionization (Fenn et al., 1989). The discovery of these two methods brought a turn around event in proteomics studies in that the analysis of proteins was improved owing to the techniques being sensitive and also the fact that they could allow analysis of large protein molecules (Chaurand et al., 1999; Siuzdak, 2004). The principle working of a mass spectrometer is that for analysis to be efficient the analytes need to be ionized or charged and these ions are transmitted through electric fields or magnetic rods and they are later directed to mass analyzers that are available in many forms.

Electrospray ionization (ESI)

Electrospray ionization is a soft ionization technique that is applicable in liquid chromatography mass spectrometry and it has become valuable in studies relating to large molecules like proteins. This technique has the ability to change solutions of analytes into gaseous form and this enhances the subsequent down stream processes (Fenn et al., 1989). The advantage of this ionization technique in proteomics studies is that it is combined with a liquid chromatography system and the proteins are transformed in such a way that the proteins possess different charges. The charged solution is usually passed through an emitter that is connected to an ion source whereby a spray is formed through the application of a huge potential difference and the ions are transferred into the mass analyzer (Smith et al., 1991).

Matrix-assisted laser desorption ionization (MALDI)

The brains behind the development of this soft ionization technique are Franz Gillenkmap and Michael Karas. They discovered that a sample mounted on a matrix which would later absorb laser radiation got ionized (Karas & Hillenkamp, 1988). Therefore, MALDI mass spectrometry is the process where peptides are laser-desorbed from a solid or liquid matrix containing a highly UV absorbing substance. The advantages associated with MALDI are that it is easy to use, sensitive and small amounts of sample are required. In addition, the matrix plays a major role of protecting the biomolecules from destruction by the laser beam. The matrix also

enhances vaporization and ionization of the peptides and therefore this technique has found application in biological analysis (Baldwin, 2005; Wall et al., 2002; Zhen et al., 2004).

2.17.6 Types of mass analysers

Mass spectrometers make use of analysers because their main role is separate ions based on their mass to charge ratio (m/z ratio). There are many analysers that are available for proteomics studies and their differences are in terms of the range of separation of the ions, they come in different sizes and sophistication, their efficiency also differs in that some give better resolution and accuracy than others. Most of the proteomics platforms improve their throughput and efficiency by carrying out the mass spectrometry analysis in conjuction with two analysers (Yates et al., 2009).

Quadrupole analyser

As the name implicates in this type of analyser, a quadrupole is formed by a connection of four parallel rods which are combined with a circular cross section. The parallel rods are then sub-divided into two rods whereby one pair is supplied with direct current (DC) while the other pair is usually supplied with alternating radio frequency (RF) and these potentials are meant to control the behaviour of the ions in the quadrupole. Through the utilization of the electric field the ions that are from the ionization chamber are able to reach the quadrupole. The instrumentation of the quadrupole is such that it has both direct current and radio frequencies in each of the rods pairs and this ensures that ions are trajected in a controlled manner. The mechanism of operation is that the DC and RF potentials cause the ions to behave differently. The posively charged ions are attaracted to the negatively charged ions but getting to the rods with radio frequency potential, the environment changes causing oscillations of the ions and therefore the ions that are able to withstand these effects are the ones that have a stable trajectory and a narrow mass to charge ratio while the rest of the ions that are less stable find their way to the rods. The combination of the different mechanisms of this kind of analyser ultimately ensures that ions bearing different mass to charge ratio find their way to the detector for analysis (Dawson, 1986; El-Aneed et al., 2009).

The type of analysers that have found application in proteomics studies are triple quadrupole analysers which are usually combined with ESI and the advantage of this set up is

that the analysers have the capability to switch between the full scan mode and the tandem mass spectrometry scan mode (McLafferty & Bockhoff, 1978; McLafferty, 1981). In essense, ions travel through the first quadrupole then move to the second quadrupole and finally through the third quadrupole to the detector. The first scan mode deals with ions that have broad range of mass to charge ratio and these ions have the ability to pass through the first quadrupole. The ions are then transmitted to the second quadrupole where the tandem mass spectrometry takes place and this analyser deals with ions that have a narrow range of mass to charge ratio and here the ions undergo fragmentation occasioned by an inert gas like nitrogen or helium. After the process of collision to fragment the ions, these ions are then allowed to pass through the third quadrupole where measurements are carried out by the detector that is connected to the set up.

Time of flight (TOF)

The principle behind this analyser is that it measures the time it takes for ions to pass through a specified path of flight. The theory behind this is that when there are ions bearing the same charge and they are passed through the analyser at the same time, they possess a similar kinetic energy but in order for the ions to get to the detector, their rate of progression is dependent on their mass to charge ratio. In the time of flight type of analysers, all the ions make their way to the detector as opposed to the quadrupole analyser where two scan modes must be carried out to filter the ions and only the fragmented ions get to the detector. The time flight analysers have a high resolution and they have found a lot of application in mass spectrometry technology (Andrews et al., 2011; Beck et al., 2015; El-Aneed et al., 2009; Gelderman & Simak, 2008).

Orbitrap mass analyzer

The brain behind the orbitrap mass analyser is Alexander Makarov (Makarov, 2000). This analyser has two electrodes and these are the central electrode and an outer electrode whose role is to trap ions and this is based on the principle that trapping of ions oscillating in orbits can exploited for various studies (Kingdon, 1923). As the ions keep rotating the central electrode they get to a point where the oscillations are harmonious and the frequency of rotation is proportional to the mass to charge ratio. The oscillations are then analysed in Fourier transform

ion cyclotron resonance (FTICR) and this type of analyser has a high resolution and is applicable in proteomics studies (Eliuk & Makarov, 2015).

Ion-Trap Mass Spectrometer

This form of mass analyser came about when it was discovered that trapped ions can be manipulated and these knowledge can be applied in proteomics studies. The theory behind this analyser is that a Scientist by the name Stafford designed an instrument that enabled the trapping of ions over a specified period of time bearing different mass to charge ratios and then these ions were later released to a detector (Stafford, 1984). This technology of trapping ions for further analysis was enhanced when ions of the same mass to charge ratio were clustered together in an ion trap facilitated by helium inert gas and the ions were later ejected at a higher rate than ions that were not clustered together. Therefore the ion trap analyser function by shuttling between aspects of ion collection, storage of the trapped ions and then release of the trapped ions and in the process peptide ions are fragmented (Cha et al., 2000). Fragmentation process only occurs to the specific trapped ions and this is because the voltage is raised causing the ions to acquire energy which allows them to vibrate and in the process the ions make collissions with helium inert gas causing the peptide ions to fragment. These ions are later released from the ion trap in a sequence depending on their mass to charge ratio. Ion traps analyse proteins in conjuction with tandem mass spectrometry whereby the full scan is used to identify certain abundant ions based on their mass to charge ratio and these are set for the fragmentation process. There are also cases where the fragmented ions are fragmented further especially proteins that have undergone post-translational modifications (March, 1997; Schwartz et al., 2002).

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR)

The first instrument of Fourier transform ion cyclotron resonance (FT-ICR) was invented in the 1970s (Comisarow & Marshall, 1974) and it has found application in mass spectrometry technologies because it has a good resolution (Amster, 1996; Dienes et al., 1996; Marshall et al., 1998; McLafferty, 1994). It involves the production of ions from a particular source that is located outside the magnetic field and the ions are directed in the cell where they are maintained by the magnetic field present in the cell (Marshall & Guan, 1996). These confined ions exhibit an orbiting mode referred to as ion cyclotron frequency. FT-ICR MS is a powerful mass analyser

providing the highest resolving power (Schaub et al., 2008), mass resolution, (Bossio & Marshall, 2002; He et al., 2001) and mass measurement accuracy, (Savory et al., 2011; Schaub et al., 2008) allowing for confident analysis of many large biomolecules including proteins (Aebersold & Mann, 2003) and nucleotides (Benson et al., 2003; Wunschel et al., 2000). The FT-ICR MS has high mass accuracy because its mode of action is to measure the frequency of the ions and then determine the mass to charge values. There are also FT-ICR instruments that have found application for commercial purposes where they work in conjunction with tandem mass spectrometry making them very useful in the study of biological systems (Zubarev et al., 1998).

Peptide fragmentation

In mass spectrometry analysis proteins and peptides have to be fragmented into ions for further analysis like tandem mass spectrometry. The process involves the selection of intact peptide ions and they are usually separated based on their mass to charge values and this usually takes place in the first mass analyser (MS1). The next process is to select the abundant protein or peptide ions and they are usually directed in a collision cell containing an inert gas like helium and interaction with this gas leads to collision and fragmentation of the ions. The fragmentation techniques that have found application in proteomics research include collision-induced dissociation, (CID) where ions are collided with neutral gas molecules like nitrogen, argon or helium and they break the ions into fragments (Cooks, 1995; Kim et al., 2010). Other ion fragmentation methods that have found application in proteomics analysis are high-energy collisional dissociation (HCD) which uses a higher voltage to collide the ions with the inert gas (Olsen et al., 2007), electron capture dissociation (ECD) and electron transfer dissociation (ETD) where a chemical reaction transfers an electron onto the ions to fragment them (Syka et al., 2004; Zubarev et al., 1998).

2.17.7 Mass spectrometry-based quantitative proteomics

There has been great advances of technology in the field of quantitative proteomics and this has ultimately led to high throughput mode of detection of analytes in mass spectrometry.There are two quantitation methods and these are absolute and relative quantification methods that are applicable in proteomics research. As the term suggests, absolute

quantification deals with the actual concentration or quantity of proteins or peptides in the analyte. On the other hand, relative quantification does more of a comparison of the proteins present in different analytes (Elliott et al., 2009). The principle behind the operation of absolute quantification is the introduction of labelled reference peptides that are of known concentration to the sample solution and then liquid chromatography coupled with tandem mass spectrometry is carried out. After the mass spectrometry analysis, the quantity of the target peptide is determined by the comparison of the intensity of the peptide of interest and that of the reference labelled peptide (Bronstrup, 2004). The draw backs associated with this mode of quantification is that it is expensive and time consuming. Relative quantification has gained a lot of popularity with most proteomics studies and it has been used to determine protein expression profiles between samples subjected to different treatments and this form of quantification can either use label free quantification methods or labelled quantification methods.

Label Free Relative Quantitation

Most the proteomics platforms make use of label free quantification as opposed to labeled forms of quantification (Wienkoop et al., 2006). Label-free quantitative proteomics (LFQP) is the most common method in proteomics because it is easier to perform especially when dealing with large sample sizes (Kalra et al., 2013; Keiji & Takashi, 2008). The advantages that go hand in hand with LFQP are that it is cheaper since the purchase of costly reagents does not apply to this technique and it is also faster to perform because no labeling strategies are undertaken (Abdalla et al., 2012). The principle behind the operation of this label free method for quantification of proteins is that a comparison is usually made between the peptides that had been initially digested by trypsin with identical peptides from different samples but whose elution took the same retention time after MS analysis. The relative concentrations of the proteins are usually determined by analysis of the peak intensities of the peptides of interest and it is important to note that the intensities of the peaks have to be normalized so that errors are minimized (Old et al., 2005; Silva et al., 2005). Therefore, label free quantitation proteomics has received a lot of acceptance as compared to the labeled strategies and as such it has found application in the analysis of many different types of biological samples (Megger et al., 2013; Wang et al., 2008b; Yan & Chen, 2005).

Stable isotope labelling techniques

The use of labelled isotopes in the quantification procedure of proteins in proteomics studies is slowly being undertaken with the advancement of mass spectrometric techniques (Tabb et al., 2016). Labelling of proteins is usually done using nitrogen, oxygen and carbon isotopes whereby labels are incorporated in metabolic reactions or by the use of enzymes (de Bievre & Taylor, 1993; Xie et al., 2011). The protein abundances are usually determined through the comparison of the peak intensities of differentially labelled peptides. There are two major labelling strategies and these are ^{15}N labelling and stable isotope labelling with amino acids in cell culture (SILAC) (Everley et al., 2004; Oda et al., 1999; Ong et al., 2002). The mode of operation of SILAC is that the basic amino acids, arginine and lysine are labelled with isotopes and then they are added in the culture medium and as the growth progresses the labels are taken up by the proteins and therefore this technique quantifies changes in proteins that occur *in vivo*. This labelling technique has found application in other organisms (Doherty et al., 2005; Gouw et al., 2010; Kruger et al., 2008; Westman-Brinkmalm et al., 2011).

The other labelling strategy is by the use of enzymes like trypsin which is used to introduce oxygen isotope into the C-terminal side of cleaved peptides because trypsin cleaves arginine and lysine amino acids at the C-terminal end (Krusemark et al., 2009; Reynolds et al., 2002; Yao et al., 2001). The disadvantages of this technique is incomplete labelling that is occasioned by the slow exchange of the two isotopes of oxygen and so peptides are labelled at different rates (Johnson & Muddiman, 2004; Ramos-Fernandez et al., 2007; Ye et al., 2009). Labelling using oxygen isotope has found application in proteomics research whereby proteins are first cleaved into peptides and then the oxygen isotope label is incorporated (Yao et al., 2001; Yates et al., 2009). There are also cases where labelling with oxygen isotope has been carried out after the digestion process (Bantscheff et al., 2004). The instruments that can accommodate the labelling strategy include the Fourier transform ion cyclotron resonance but research has shown that even instruments like ion traps can also be used (Heller et al., 2003). Chemical reactions can also be used to introduce labels comprising of tags to protein or peptides (Desiderio & Kai, 1983). For chemical labelling the strategy involves the use of isotopic and isobaric tags for labelling, e.g. isotope coded protein labelling (ICPL) (Munchbach et al., 2000), isotope-coded affinity tag (ICAT), isotope tags for relative and absolute quantification (iTRAQ), tandem mass tags (TMT) and stable-isotope dimethyl labelling (DML).

Isotope-Coded Affinity Tag (ICAT)

This is an *in-vitro* isotopic labelling method for proteins and it is used for the quantification of proteins. The composition of the chemical reagent consists of an iodoacetamide, a linker region containing the heavy or light label and a biotin residue for peptide enrichment using affinity chromatography (Smolka et al., 2001). The procedure involves the incubation of the samples with the 'light' or 'heavy' label and this enhances the binding of sulfhydryl groups of cysteine resides through the iodoacetamide region of the ICAT molecule. After the samples have been labelled, they are then mixed, digested and from there the peptides that specifically contain cysteine are enriched using affinity chromatography through the binding of the biotin label to avidin coated stationary phase. This technique is sensitive and specific and it has found application in all sample types (Qu et al., 2006).

Isobaric Tags for Relative and Absolute Quantification (iTRAQ)

This quantification method uses tags that must have equal masses but during dissociation they give rise to different ions (Thompson et al., 2003). The use of isobaric tags for labelling has found application in comparative proteomics. One of the advantages of this technique is that it is a high through-put technique and as many as eight samples can be analysed simultaneously in a single experiment provided the tags of the reporter group are different from those of the balancer group (Choe et al., 2007; Ross et al., 2004). Isobaric tags for relative and absolute quantification (iTRAQ) have found application for comparison of protein expression levels in various organisms (Moulder et al., 2018; Ross et al., 2004; Thompson et al., 2003).

2.17.8 Peptide Mass Fingerprinting (PMF)

Peptide mass fingerprinting (PMF) is a technique that is used for the identification of proteins that have been separated using two-dimensional gel electrophoresis (Henzel et al., 1993). Proteins are cleaved using specific enzymes like trypsin that cleaves the amino acids lysine and arginine at the C-terminal end (Olsen et al., 2004; Wilkinson, 1986). The peptides are analysed for mass determination and quantification through the mass spectrometry platform. To identify the peptides, a suite of Bioinformatics tools are used to match the peptides in the samples with those present in the data bases.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Establishment of cadmium tolerant and control untreated colony of *An. gambiae s. s*

Anopheles gambiae s. s mosquitoes were collected from a colony at the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya, in the animal rearing and quarantine unit (ARQU). *Anopheles gambiae s. s* mosquitos were collected previously from Mbita field station (00.025'S, 34.013'E) in Homa Bay County, Kenya. The mosquito colony was raised in specially built, climate-controlled rooms. The temperature in the insectary was constantly maintained at 28±2°C. The relative humidity was maintained at 75%-80% especially for the adults and this was made possible by a mounted humidifier. Adult mosquitoes are harmed by excessively high humidity, which must be avoided (Benedict, 1997; Gerberg et al., 1994). Lighting was set at 12:12 light: dark schedule and this is the commonly applied lighting schedule in most insectaries (Mireji et al., 2010b). Selection of cadmium tolerance in juvenile stages of *An. gambiae s. s.* was done in triplicates through exposure to cadmium metal whose concentration had been emphirically determined for the mosquito. Mosquito's first three stages of development were exposed to cadmium metal solution at a concentration of 0.36µg/L. The larvae were reared in 1500mL of cadmium metal solutions and in the same way, control population of *An. gambiae s. s* was reared in triplicates in clean distilled water that was devoid of cadmium heavy metal. After every 24 hours, water was changed and clean distilled water for the control group was added while clean cadmium metal solution was added for the cadmium treated group. This procedure was repeated until pupae of the respective triplicate groups emerged. Pupae were collected and put in cylindrical pans and sealed with a net to prevent mosquitoes from flying away. The pupae were transferred to a separate room awaiting adult emergence. The colony was held in good working order by observing the rules and regulations required for mosquitos rearing. Pulverized tetramine fish food was fed to the larvae stages (Tetra GmbH, Melle, Germany). The adults were fed a ten percent sucrose solution. Female mosquitos were fed blood from anaesthetized mice in order to lay eggs (Ford & Green, 1972). The Kenya National Ethical Review Board approved the feeding of mosquitoes to mice (protocol number KEMRI/RES/7/3/1) and KEMRI's Animal Care and Use Committee (ACUC) reviewed the protocol. The colony was in the 90th filial generation at the time of this work,

and by the time the research was finished, it had grown to the 120th filial generation. The third instar larvae of the respective triplicate control and cadmium treated groups were used in this study. Cadmium was used in this analysis as cadmium chloride ($CdCl_2$), which was 99.99 percent pure (Fisher Scientific LLC, Fair Lawn, NJ, U.S.A.).

3.2 RNA extraction

The ZR Tissue and Insect RNA Microprep kit™ (ZR Tissue and Insect RNA Microprep kit™) or the Trizol reagent (Invitrogen, Carlsbad, CA) is used to isolate total RNA (Zymo Research Corporation, Irvine, CA, U.S.A.). Both methods of RNA extraction were used at first, but when the kit ran out, the Trizol reagent became the primary mode of RNA extraction in this analysis. However, both methods generated RNA of comparable quality.

3.2.1 RNA extraction using ZR tissue and insect microprep kit

Complete RNA was isolated from the third instar larvae of both the cadmium-treated and control strains. The procedure was as described below: Thirty larvae samples were placed in a BashingBead lysis tube, 800 µl RNA lysis buffer was applied, vortexed, and centrifuged at 12,000 rpm for one minute. 400µl of the supernatant was centrifuged at 8,000 rpm for 30 seconds on a Zymo-spin IIIC column in a collection tube. A total of 0.8 volume of ethanol was applied to the flow through, which was thoroughly mixed before being moved to a Zymo-spin IC column in a collection tube and centrifuged for 30 seconds at 12,000 r.p.m. The flow through was thrown out. A 400µl RNA prep buffer was applied to the column and centrifuged for 1 minute at 12, 000 r.p.m. The Zymo-spin IC column was replaced back into the collection tube after the flow through was discarded. The column was washed with RNA wash buffer (800µl) and centrifuged for 30 seconds at 12,000 rpm. The Zymo-spin IC column was replaced back into the collection tube after the flow through was discarded. The wash cycle was repeated with half the volume of RNA wash buffer, followed by 2 minutes of centrifugation at 12,000 rpm. The Zymo-spin IC column was carefully removed from the collection tube and inserted into a tube that was free of DNase and RNase. 6µl of DNase/RNase-free water were poured directly into the column matrix and left to stand for 1 minute. To elute the RNA from the column, centrifugation at 12,000 rpm for 30 seconds was used. Extracted RNA was either used right away or deposited in the deep freezer at -80°C.

3.2.2 RNA extraction using the Trizol reagent

Using the Trizol RNA isolation reagent, total RNA was isolated from the third instar larvae of cadmium heavy metal tolerant and control untreated *An. gambiae s. s* populations (Invitrogen, Carlsbad, CA). In a nutshell, 30 larvae samples were transferred to l.5mL RNase-free microcentrifuge tube. Trizol^{TM} reagent (750µl) was added, thoroughly mixed by hand shaking, and incubated for 10 minutes at room temperature. The tubes were filled with 200µl of chloroform and tightly capped before vortexing for 15 seconds. The tubes were incubated for 10 minutes at room temperature before being centrifuged for 10 minutes at 4°C at 12,000 r.p.m. After phase separation with Trizol/chloroform, the upper colorless aqueous phase was transferred to a new 1.5mL RNase-free micro-centrifuge tube containing 500µL isopropanol and 1µl of 50mg/mL glycoblue without interfering with the DNA interface. The samples were vortexed for 15 seconds and then incubated for 10 minutes at room temperature before being centrifuged at 12,000 rpm for 10 minutes at 4°C. Without losing the obvious blue RNA pellet, the supernatant was pipetted out. The RNA pellet was washed on ice with 500µl of 75 percent ethanol, vortexing to mix and centrifugation at 12,000 rpm for 2 minutes at 4°C. The supernatant was removed and the RNA pellet was dried for a few minutes. After that, the RNA pellet was dissolved in 20µl of nuclease-free water. The RNA samples were immediately quantified by reading the absorbance at 260/280 nm. Glycoblue is an inert co-precipitant made up of a blue dye covalently linked to glycogen that is free of nucleic acids, DNases, and RNases pollutants. GlycoBlue^{TM} increases RNA isolation yield while having no impact on the efficiency of isolated RNA or 260/280 reading quantitation. It also doesn't interact with or compete with enzymatic reactions that follow.

3.3 DNase I digestion

To extract the residual DNA from the isolated total RNA, DNase I (RNase free; TaKaRa) was used. The reaction buffer, DNase I and RNase inhibitor were mixed with the RNA and incubation was performed at 37°C for 30 minutes. Then 1µL 50Mm EDTA was added and incubation was performed at 65°C for 10 minutes. The purified RNA obtained was quantified and then storage was at -80°C in a deep freezer or in liquid nitrogen until use.

3.4 RNA Quantification

The concentrations of the total RNA were determined using either a micro-spectrophotometer Genequant pro (Amersham Pharmacia Ltd., Bucks, UK) or a NanoDrop® (Thermo Scientific) depending on their availability.

3.5 Gene Fishing™ Reverse Transcription

The Annealing Control Primer (ACP) technology was chosen for this study because it enhances the precision and sensitivity of PCR amplification and produces only specific PCR products. In addition, PCR products can be detected on regular ethidium bromide-stained agarose gels and ACP needs only a small amount of starting content (Hwang et al., 2004). However, this technology has lower throughput than other competing technologies such RNA sequencing. Complete RNA was extracted from both the cadmium heavy metal tolerant population and the control *An. gambiae s. s.* population, standardized to equivalent concentrations and used directly for reverse transcriptase-mediated synthesis of first strand cDNA (Hwang et al., 2003). In a final reaction volume of 20 µL, reverse transcription was conducted for one and a half hours at 42°C with 2µg of RNA, 4µl of 5X reaction buffer (Promega, Madison, WI, USA), and 5µl of dNTPs (2mM each of dATP, dCTP, dGTP, dTTP), 2 µl of 10 µmol cDNA synthesis primer dT-ACPl (5'-CGTGAATGCTGCGACTACGAT1IIII$(T)_{18}$-3'), Moloney murine leukemia virus reverse transcriptase (200 U/L, Promega) and 0.5 L of RNasin® RNase Inhibitor (40 U/L, Promega). For the Gene Fishing PCR, the synthesized first-strand cDNA was diluted with 80µl ultra-purified water and processed at -20°C before use.

3.6 Annealing Control Primer-based gene fishing™ PCR

The Gene Fishing DEG kits were used to screen differentially expressed genes (DEGs) using the Annealing Control Primer (ACP)-based PCR process (Seegene, Seoul, South Korea) (Kim et al., 2004a). In a single tube, second-strand cDNA synthesis and subsequent PCR amplification were carried out. Second-strand cDNA was synthesized in a final reaction volume of 20µl containing 3-5µl (about 50ng) of diluted first-strand cDNA during one step of first-stage PCR at 50°C, l µl of dT-ACP2 (10mM, reverse primer) (5'-CTGTGAATGCTGCGACTACGATIIIII $(T)_{15}$-3', l µL of 10 µM forward primer and 10µl of 2x master mix. For second strand synthesis, the PCR protocol consisted of one cycle at

94°C for 1 minute, followed by 50°C for 3 minutes, and 72°C for 1 minute. The second-stage PCR amplification protocol was 40 cycles of 94°C for 40 seconds, followed by 65°C for 40 seconds, 72°C for 40 seconds and 10 minutes final extension at 72°C after second-strand DNA synthesis was completed. The PCR products were isolated on a 2% agarose gel in Tris-Borate EDTA buffer for one hour at 80 volts and stained with ethidium bromide.

3.7 Gel extraction

The QIAquick® Gel extraction kit was used to remove the gels (QIAGEN Inc., Valencia, CA). Using a scalpel, differentially expressed bands were excised from the agarose gel between the control and the cadmium heavy metal tolerant populations and UV light was used. Three volumes of buffer QX1 were applied to one volume of the gel slice after it was weighed. For 10 minutes at 50°C, the mixture was incubated until the gel slice was fully dissolved. Vortexing was performed every 3 minutes during the incubation period to help dissolve the gel. One volume of isopropanol was applied to the sample and combined after the gel slice was fully dissolved as was shown by the yellow hue. In a 2 mL collection tube, the QIAquick spin column was mounted. The sample was added to the QIAquick column and centrifuged for 1 minute to bind DNA. The flow through was thrown away and the QIAquick column was reinserted into the same collection channel. 0.75mL of buffer PE was applied to the QIAquick column and centrifuged for 1 minute to complete the washing process. The flow through was discarded and the QIAquick column was centrifuged at 13,000 rpm for another minute. In a clean 1.5 mL microfuge tube, the QIAquick column was put. 30µl of elution buffer was applied to the centre of the column for DNA elution, the set up was allowed to stand for 1 minute and then centrifuged for 1 minute at 13,000 r.p.m.

3.8 Cloning

The gel filtered products were directly cloned into Invitrogen's pGEM-T Easy vector (Carlsbad, CA, USA) and transformed into JM109 competent cells. For blue/white colony selection, colonies from competent cells were grown for 18 hours at 37°C on Luria broth agar plates containing ampicillin, X-gal (5-bromo4-chloro 3-indoy1-D-galactopyranoside) and isopropyl-D-thiogalactopyranoside (IPTG). The GeneJET^{TM} plasmid Miniprep kit was used to purify the transformed plasmids (Fermentus, Thermo Fisher Scientific Inc). In a nutshell, cells

were suspended in 250µl of resuspension solution then 250µl of lysis solution were applied and mixed by inverting the tube about five times until the solution was viscous and partially clear. Then 350 µl of neutralization solution was applied and thoroughly combined by inverting the tube for about five times. To pellet debris and chromosomal DNA, centrifugation at 6,800 rpm for 5 minutes was used. The supernatant was moved to the GeneJet spin column, but the white precipitate was not transferred. The flow through was discarded and the column was returned to the same collection tube after centrifugation at 6,800 rpm for 1 minute. The wash solution (500µl) was applied to the column and centrifuged for 1 minute at 6,800xg, the flow through was discarded and the wash phase was repeated once more.The flow through was discarded and the residual wash solution was removed by centrifugation for an additional minute. To elute the plasmid DNA, the column was moved to a new 1.5mL microfuge tube and 50µl Elution buffer was applied to the middle of the GeneJet column. After 2 minutes of incubation at room temperature, the samples were centrifuged for 2 minutes at 6,800 r.p.m. The filtered plasmid DNA was deposited at -20°C before shipping and the column was discarded.

3.9 Sequencing

For sequencing, the purified plasmid DNA samples were sent to Macrogen in Korea. M13 Primers were used for sequencing on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The raw sequences obtained were subjected to JustBio software to clean the sequences and remove any gaps present before pasting the sequences to VecScreen to get the positions that are contaminated on the vector and that serves as the insert sequence or the sequence of interest. The sequences of interest were then edited using BioEdit software. Using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) search software, complete sequences were compared in VectorBase against *Anopheles gambiae* PEST strain transcript sequences, AgamP4.6 geneset.

3.10 Protein expression studies

3.10.1 Test insects

The insects used for proteomics work were raised in triplicates in cadmium metal and the controls were not subjected to heavy metal treatment. Mosquito propagation and tolerance experiments were done as previously explained.

3.10.2 Protein extraction

Proteins were extracted from triplicate samples (n=50) using the Trizol reagent. In a nutshell, the flow through mixture containing proteins and other mosquito tissues was precipitated using 1 mL of isopropanol per 750µL sample, incubated for 10 minutes at room temperature and centrifuged for 10 minutes at 12,000 rpm at 4°C. The recovered pellet was washed three times in 0.3M guanidine hydrochloride in 100 percent ethanol, with each wash taking 20 minutes and the pellet recovery being undertaken by centrifugation at 12,000 rpm for 10 minutes. After the final wash the pellet was allowed to dry and it was then re-suspended in 100 µL of SUBT buffer (4.5 M urea in 0.5% SDS, 25 mM Tris/HCl, pH 7.5) (Bai and Laiho, 2012) and protease inhibitors were incorporated. Protein quantification was performed using Bradford assay (Bradford, 1976).

3.10.3 Liquid chromatography and MS/MS of *An. gambiae s. s* larvae proteome

LC-MS/MS analysis was carried out following the protocols designed by Njunge et al., (2017). Briefly, equal amounts of the sample proteins were diluted in Laemmle sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% B-mercaptoethanol, 0.01% Bromophenol blue), the resultant solution was boiled and the inherent protein was separated on a 12% acrylamide sodium dodecylsulphate-polyacrylamide gel electrophoresis (NuPAGE 4-12% Bis-Tris Gel, Life Technology) in triplicates. Methanol/acetic acid were used to fix the gel and then Coomassie brilliant blue (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) was used for staining. The gel was destained in methanol as previously described (Njunge et al., 2017). Each gel lane was cut into six parts and destained in a 50 mM ammonium bicarbonate/50 percent CH3CN solution. The gel fragments were digested overnight at 37°C using the trypsin enzyme (Thermo Fisher Scientific). The peptides were then extracted in 0.5 percent formic acid (FA)/50 percent CH3CN and dried in a SpeedVac (Thermo Fisher Scientific). Just before the LC-MS/MS review, the peptides were resuspended in 20µl of 0.5 percent FA. Peptides (5µL) were loaded onto a 75 m x 2 cm C18 trap column (Thermo Scientific) and separated on a 75 pmx 25 cm C18 reverse-phase analytical column (Thermo Scientific) using a Dionex Ultimate 3000 nano-flow ultra-high-pressure liquid chromatography system (Thermo Scientific). Over 120 minutes, elution was carried out with mobile phase B (80 percent acetonitrile with 0.1 percent formic acid) gradient (5-35%). An Exactive Orbitrap mass spectrometer (Thermo Scientific) was used to

calculate peptides, which was connected to the chromatography device through a nano-electrospray ion source (Thermo Scientific). Resolution: 70,000; AGC target: 3e6; scan range: 400-1800 m/z for ms1; Resolution: 17,500; AGC: 5e4; isolation window: 1.6 m/z for ms2. For ms2, the top 15 most intense ions were chosen and they were then omitted for the next 30 seconds.

For both the peptide-spectrum match level calculated by the target-decoy approach and the protein FDR, the false discovery rate was set at (FDR=0.01) 1%. MaxQuant (Version 1.5.3.30, www.MaxQuant.org) was used to process the raw files and search them against a combined database of *Anopheles gambiae* proteins (Agamp4, from Vectorbase) and a contaminate database provided by MaxQuant. With the dataset identifier PXD010707, the mass spectrometry proteomics data has been deposited in the ProteomeXchange Consortium through the PRIDE (Deutsch et al., 2017; Vizcaino et al., 2014) collaborator repository.

3.10.4 Differential protein abundance analysis

Label free quantification (LFQ) of proteins was performed using MaxQuant software (Version1.5.3.30, www.MaxQuant.org) to classify differentially expressed proteins. LFQ values were normalized for all replicates (Cox & Mann, 2008; Kaur et al., 2017) and used for analysis. The mean protein expression values per group for each protein were obtained and used to determine differential protein abundance. The changes in protein abundance were computed from the difference in log2 values in (replicate-mean). Unpaired student t-test was performed on the mean protein abundance between the cadmium-tolerant and the control groups to identify proteins whose abundance were significantly altered. After applying the Benjamini Hochberg (BH) correction to the p-values, proteins with a modified p value of $p < 0.05$ were deemed important for further study. Principal Component Analysis (PCA) was used to examine patterns of variation among samples. The global changes involving biological processes were investigated using STRING database (Szklarczyk et al., 2011) to identify known protein-protein interactions (PPIs) among the differentially expressed peptides using orthologs of the *An. gambiae* proteins in *Drosophila melanogaster*.

CHAPTER FOUR

RESULTS

4.1 Establishment and maintenance of cadmium tolerant and control colony

A colony of cadmium tolerant and control non-exposed mosquitoes was generated in triplicates in the insectary at ICIPE following the standard protocols of colony establishment and maintenance. The third instar larval stage of both populations was used for this study.

4.2 RNA Quantification

Total RNA was extracted from the third instar larvae of cadmium heavy metal tolerant and control *An. gambiae s. s* populations in triplicates using Trizol reagent (Invitrogen) or the ZR Insect and Tissue RNA kit™ (Zymo Research Corporation, Irvine, CA, USA) depending on the reagent availability. The micro-spectrophotometer Genequant pro (Amersham Pharmacia Ltd, Bucks, UK) was used to quantify the extracted RNA and a few samples were quantified using the NanoDrop® (Thermo-Scientific) (Table 1 and 2). When the micro-spectrophotometer developed a mechanical problem, the NanoDrop® was used only once for quantification.

Table 1: RNA Quantification using the micro-spectrophotometer of cadmium and control samples extracted using either Trizol reagent or ZR Insect and Tissue RNA kit.

Sample Name	A260/A280	Conc (µg/ml)	Extraction method
CTI	1.953	351.51	Trizol Reagent
CT2	1.972	341.21	Trizol Reagent
CT3	1.96	2115.2	ZR Insect and Tissue RNA kit
CT4	2	3189.2	ZR Insect and Tissue RNA kit
CDl	1.923	298.63	Trizol Reagent
CD2	1.962	300.34	Trizol Reagent
CD3	2	1824.2	ZR Insect and Tissue RNA kit
CD4	2	3061.2	ZR Insect and Tissue RNA kit

Table 2: RNA Quantification of the control samples (CT) and cadmium tolerant samples (CD)

Technique	Sample ID	A260/A280	RNA Conc µg/mL)
NanoDrop®	CT1	1.96	211.52
	CT2	2.00	318.92
	CD1	2.00	182.42
	CD2	2.00	3061.20
Micro-spectrophotometer	CT10	1.89	236.04
	CT11	1.84	180.96
	CT12	1.86	153.52
	CT13	1.90	264.65
	CT14	1.85	189.21
	CT15	18.49	241.36
	CT16	1.87	230.27
	CT17	1.88	158.25
	CT18	1.84	188.18
	CT19	1.87	190.38
	CT10	1.93	296.83
	CT11	1.90	240.82
	CT12	1.91	236.82
	CT13	1.93	225.05
	CT14	1.93	283.98
	CT15	1.85	187.16
	CT16	1.85	244.09
	CT17	1.92	252.15
	CT18	1.96	322.71
	CT19	1.90	389.50

Since the package could not handle many samples and eventually became depleted, the majority of the RNA extractions in this study were done with the Trizol reagent (Invitrogen). The extracted RNAs were found to be appropriate for downstream use in GeneFishing Technology because the content of the RNA obtained from both methods met the purity criteria.

4.3 Differentially expressed gene transcripts in *An. gambiae s. s*

To identify genes that were differentially expressed in cadmium tolerant *An. gambiae s. s* larvae, mRNA expression profiles of cadmium-treated larvae were compared with those of the control (untreated larvae) by the application of the GeneFishing technique with ACP system (Kim *et al.*, 2004a). Through the ACP system, fourteen (14) differentially expressed genes (DEGs) were identified. The DEGs were detected on the agarose gel (Fig. 4) using randomly chosen arbitrary ACP primers. BLAST searches of DEG sequences against *Anopheles gambiae* PEST strain transcript sequences, AgamP4.6 geneset, were performed in VectorBase. Table 3 lists some genes that were found to be differentially expressed in response to heavy metal exposures.

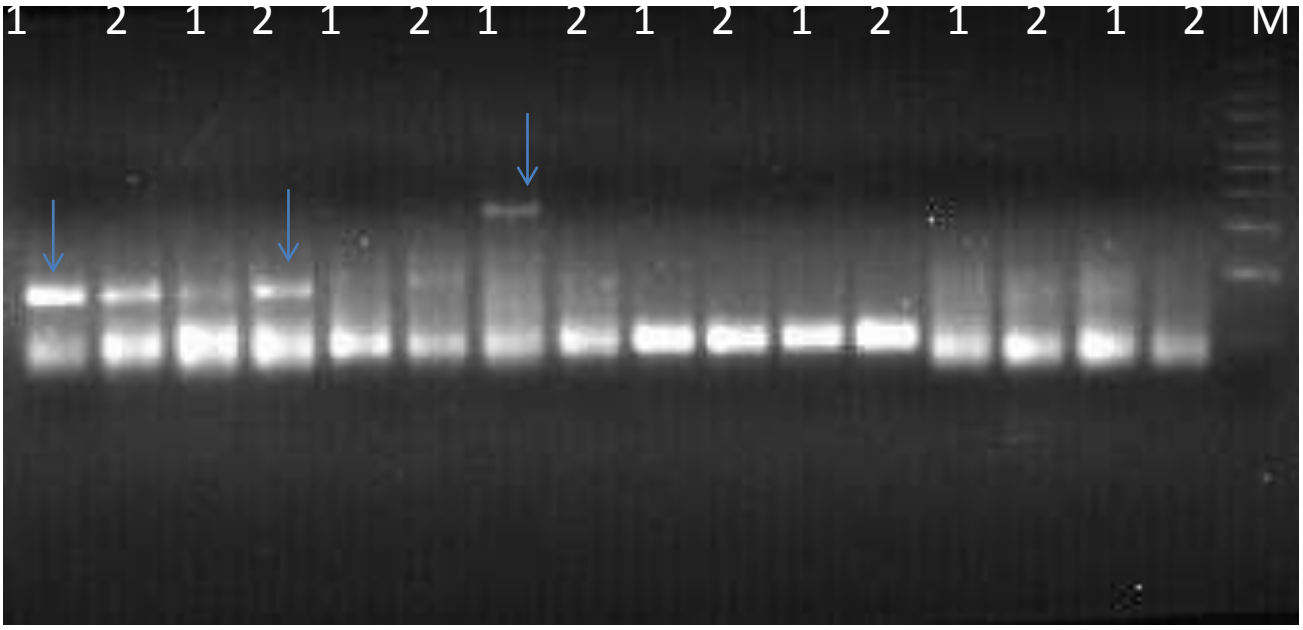


Figure 4: Differential banding patterns in mosquito larvae from cadmium-treated and control populations. DEGs were detected using the ACP 75, ACP 76 and ACP 78 primer sets, as shown by the arrows. The number 1 denotes a cadmium-treated population, while the number 2 denotes a control population. M is a molecular size marker of 50 bp.

Table 3: Gene transcripts obtained through BLAST searches from vector Base.

Gene ID	Gene name	Description of gene product	E-Value	% ID	Expression pattern
AGAP002638	ABCH1	ATP-binding cassette transporter (ABC transporter) family H member 1	3	77.5	Up
AGAP001249		Eupolytin	3e-31	98.7	Up
AGAP028915	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	8e-79	98.2	Up
AGAP004750		Translation initiation factor 4G	6.4	87	Up
AGAP028915	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	8e-78	99.4	Up
AGAP006187		Protein G12	6.8	100	Down
AGAP003078		Endoplasmic reticulum metallopeptidase 1	1.5	80.6	Down
AGAP028391	lsu rRNA		3e-103	100	Up
AGAP028915	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	4e-49	96.6	Up
AGAP028915	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	5e-81	98.8	Up
AGAP003870	Thoc7	THO complex subunit 7	6.4	87	Up
AGAP008584		lysosomal alpha-mannosidase	3.4	90.5	Up
AGAP010252	RpL23	60S ribosomal protein L23	4e-12	100	Up
AGAP028907	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	3e-06	91.2	Up
AGAP02818	5_8S_rRNA 5.8S ribosomal RNA	5.8S ribosomal RNA	3e-37	98.9	Up
AGAP028899	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	2e-08	100	Up
AGAP009563		myotubularin related protein 2	0.74	91.3	Up
AGAP002262		adenylate cyclase 8	9.6	100	Down
AGAP012302		Sodium-independent sulfate anion transporter	0.36	88.9	Up

Over 70% of genes that were identified using the ACP system were up-regulated and these included ATP-binding cassette transporter, Eupolytin, Eukaryotic small subunit ribosomal RNA, Translation initiation factor 4G, THO complex subunit 7, lsu rRNA, lysosomal alpha-mannosidase, 60S ribosomal protein L23, 5.8S ribosomal RNA, myotubularin related protein 2 and Sodium-independent sulfate anion transporter. The down regulated genes that were detected included adenylate cyclase 8, Protein G12 and Endoplasmic reticulum metallopeptidase 1.

4.4 Protein Expression in *An. gambiae s.s* larvae

A total of 1067 distinct mosquito proteins were found in both the cadmium and control mosquito groups (FDR0.01). This accounts for about 8% of the predicted proteins in the *An. gambiae* genome. 745 proteins were found in both groups, while 322 proteins were found either in the cadmium (31 proteins) or control groups (291proteins) (Fig. 5).

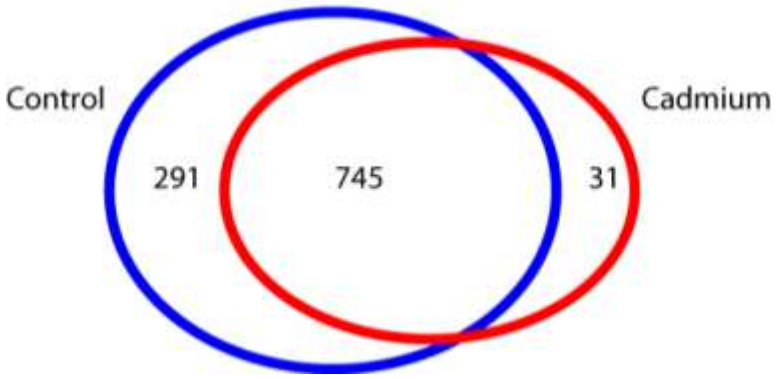


Figure 5: Mosquito L3 larvae proteins found in both cadmium and control populations, as well as those specific to each, are depicted in the Venn diagram

Principal Component Analysis on the replicate samples distinguished the cadmium tolerant larvae from the control population (Fig. 6).

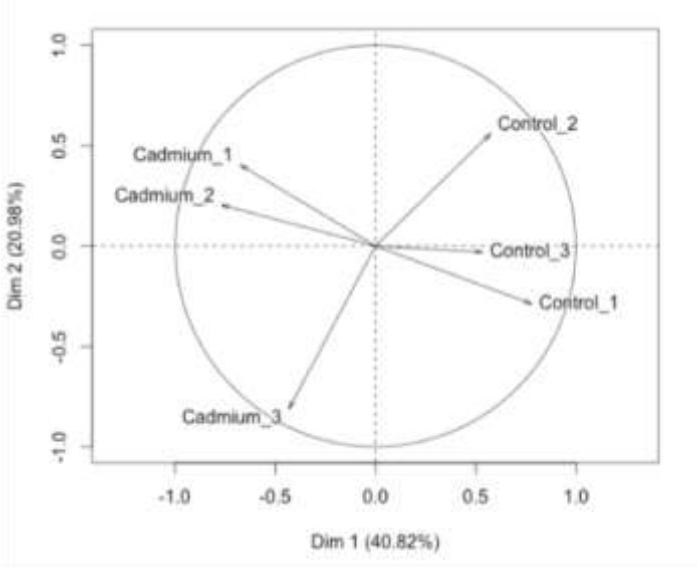


Figure 6: Principal Component Analysis (PCA) of the replicate samples for Cadmium tolerant versus Control group.

To determine protein signatures associated with Cadmium tolerance, differential protein abundance analysis was performed based on the LFQ values and revealed 63 proteins that were differentially expressed (Table 4). The proteins that were up-regulated were 18 and they included oligosaccharyltransferase complex subunit, ribose 5-phosphate isomerase A, 40S ribosomal protein S23, eupolytin and initiation factor among others. There were 45 down-regulated proteins which consisted of glucosyl/glucuronosyl transferases, clip-domain serine protease, paramyosin, NADH dehydrogenase (ubiquinone) 1 alpha and 5-methylthioadenosine phosphorylase among others. There were also hypothetical proteins that were differentially expressed.

Log2 value of protein expression (replicate-mean) for each protein was calculated and 2-tailed student t-test calculated in cadmium tolerant versus control population. Proteins with BH corrected p value < 0.01 were used for generating heatmap in R Bioconductor heatmap.2 package (Figure 7).

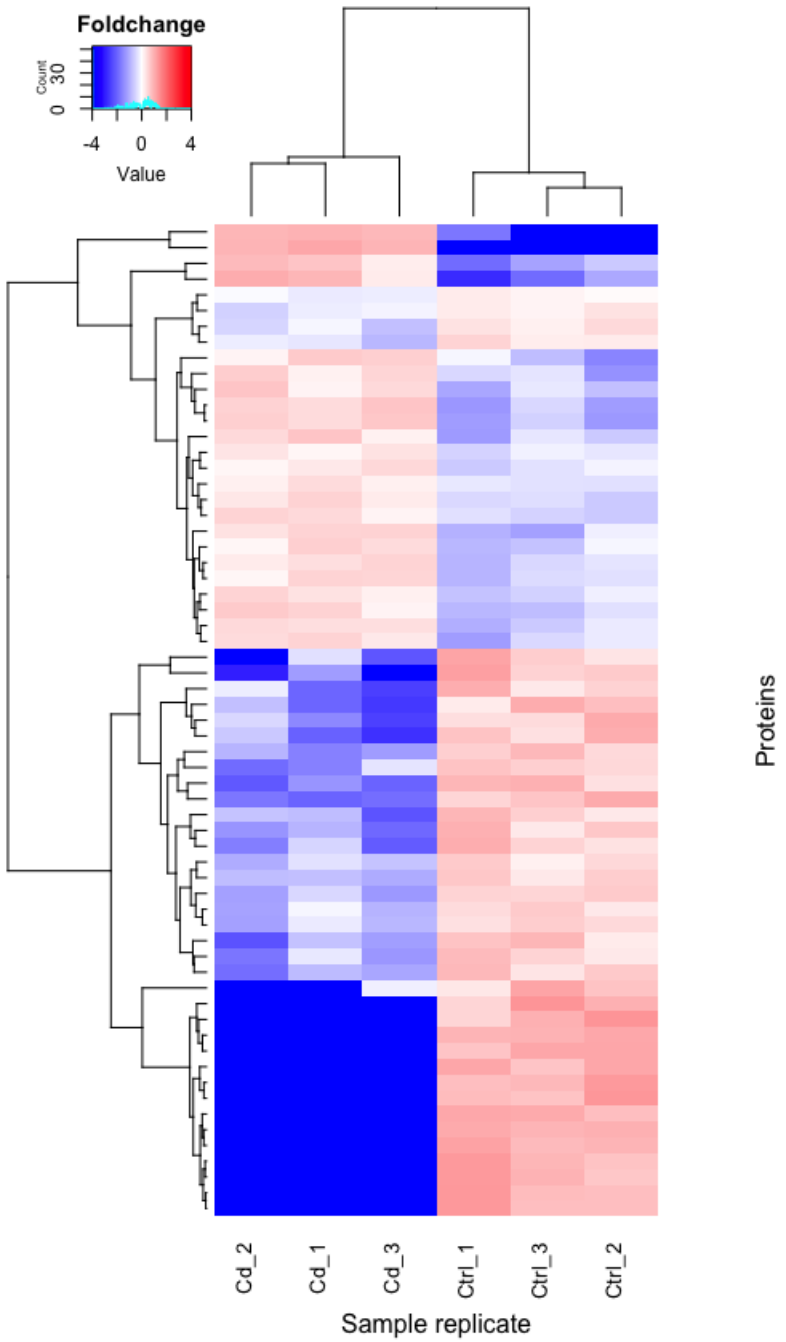


Figure: 7 Heat map illustrating proteins differentially expressed in cadmium treated population versus the control group

Table 4: Diff erentially expressed *An. gambiae s. s* larval proteins after exposure to cadmium heavy metal

Protein_ID	Gene.name	Description	l2 fold	BH. Corrected P value
AGAP005163-PA		Glucosyl/glucuronosyl transferases	< -7.9	2.34E-04
AGAP028204-PA		hypothetical protein	2.56	4.69E-04
AGAP000315-PA	CLIPC6	Clip-domain serine protease	−6.38	7.03E-04
AGAP004877-PB		paramyosin	< −7.8	9.37E-04
AGAP010174-PA		oligosaccharyltransferase complex subunit	2.37	1.17E-03
AGAP000399-PA		squid	−1.24	1.41E-03
AGAP013112-PA		mRNA binding protein	−0.5	1.64E-03
AGAP004514-PA		nuclear GTP-binding protein	< −7.7	1.87E-03
AGAP008499-PA		Mitochondrial transcription factor A	1.74	2.11E-03
AGAP004395-PA		nucleophosmin 3	< −7.6	2.34E-03
AGAP013365-PA		hypothetical protein	−0.88	2.58E-03
AGAP004895-PA		carbonic anhydrase	< −7.5	2.81E-03
AGAP003216-PA		26S proteasome regulatory subunit T2	< −7.4	3.05E-03
AGAP013036-PA		hypothetical protein	1.58	3.28E-03
AGAP011802-PA	RpL39	60S ribosomal protein L39	−1.99	3.51E-03
AGAP012317-PA		hypothetical protein	−1.84	3.75E-03

AGAP007250-PA		hypothetical protein	−2.85	3.98E-03
		cuticular protein (putative)		
AGAP028178-PA	CPLCP13	CPLCP13	−0.74	4.22E-03
		Mitochondrial ribosomal		
AGAP007507-PA	mRpL48	protein L48	−7.35	4.45E-03
		5-methylthioadenosine		
AGAP005129-PA		phosphorylase	< −7.3	4.69E-03
AGAP011298-PA	RpL10a	60S ribosomal protein L10a	−0.18	4.92E-03
AGAP004606-PA		HYPK	< −7.2	5.15E-03
		Clip-Domain Serine		
AGAP004719-PA	CLIPC9	Protease	< −7.1	5.39E-03
		ribose 5-phosphate		
AGAP011457-PA		isomerase A	1.82	5.62E-03
		protein disulfide isomerase		
AGAP007393-PC		family A, me	−0.69	5.86E-03
		Prophenoloxidase		
AGAP010730-PA		activating factor	−0.7	6.09E-03
AGAP012504-PA		hypothetical protein	0.36	6.33E-03
AGAP004618-PA		hypothetical protein	< −7.0	6.56E-03
AGAP004015-PA	SP21408	prolylcarboxypeptidase	< −7.1	6.79E-03
		protein disulfide-isomerase		
AGAP012407-PA		A1	2.09	7.03E-03
AGAP011476-PA		hypothetical protein	−2.7	7.26E-03
AGAP013347-PA		hypothetical protein	−0.74	7.50E-03

AGAP007060-PA		hypothetical protein	−3.32	7.73E-03
AGAP009216-PA		Clip-domain serine protease	−2.52	7.97E-03
AGAP007249-PB	Flightin	Flightin	−1.6	8.20E-03
AGAP010392-PA		Calumenin	−1.02	8.43E-03
AGAP011276-PA		hypothetical protein	−0.17	8.67E-03
AGAP011477-PA		Eupolytin	1.02	8.90E-03
AGAP007666-PA		Calcyphosin-like protein	−1.21	9.14E-03
AGAP009652-PA		NADH dehydrogenase (ubiquinone) 1 alpha	−2.13	9.37E-03
AGAP010181-PA		obelix	−2.42	9.61E-03
AGAP004443-PB		glycogen synthase kinase 3 beta	< -7.2	9.84E-03
AGAP012990-PA	RpS23	40S ribosomal protein S23	0.22	1.01E-02
AGAP008440-PA		urate oxidase	−5.33	1.03E-02
AGAP010404-PB	GSTS1	glutathione S-transferase	1.54	1.05E-02
AGAP009863-PA		Eukaryotic translation initiation factor	0.99	1.08E-02
AGAP010613-PA		elongation factor 1-beta	−1.16	1.12E-02
AGAP010100-PA	CPR84	cuticular protein 84 RR-2 family	1.06	1.15E-02
AGAP009271-PA	Prosbeta1	20S proteasome subunit beta 1	−3	1.17E-02
AGAP012823-PA		NADH dehydrogenase 1 alpha subcomplex su	1.8	1.19E-02

AGAP012056-PA		cofilin	0.3	1.22E-02
AGAP006469-PA		cell growth-regulating nucleolar protein	−2.51	1.24E-02
AGAP013185-PA		hypothetical protein	1.62	1.27E-02
AGAP010477-PB		phosducin-like 3	−4.57	1.29E-02
AGAP005559-PA		26S proteasome regulatory subunit N10	0.04	1.31E-02
AGAP012425-PA		hypothetical protein	1.91	1.34E-02
AGAP012837-PA	mRpL43	39S ribosomal protein L43	−1.67	1.36E-02
AGAP005696-PA	CPTC1	cuticular protein from two-cysteine fami	−7.04	1.38E-02
AGAP007172-PB		translation initiation factor 4E	−2.43	1.41E-02
AGAP010731-PA	CLIPA8	Clip-Domain Serine Protease	−1.15	1.43E-02
AGAP003360-PA		dynein light chain roadblock-type	< −7.3	1.45E-02
AGAP011098-PA		beta-aspartyl-peptidase (threonine type)	2.15	1.48E-02
AGAP006117-PA		hypothetical protein	< −7.4	1.50E-02

4.4.1 Functional enrichment and network analysis

Functional enrichment was performed by STRING online database using KEGG pathways and 14 down-regulated pathways were identified. Among them were pathways linked to oxidative phosphorylation, glycolysis and nitrogen (arginine and proline) metabolism. The y-axis shows biological pathways that were significantly altered based on the –log 10 FDR values plotted in the x-axis (Figure 8). Enrichment was only observed in down-regulated proteins.

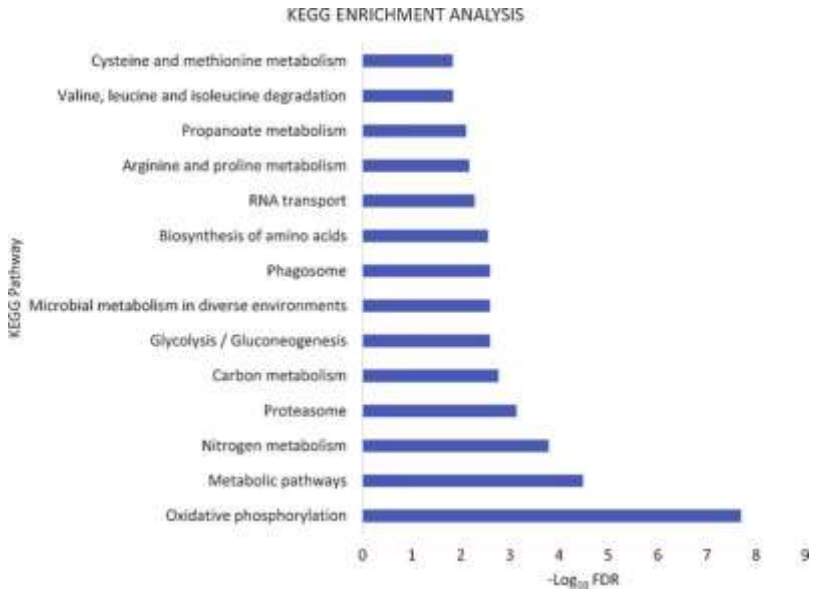


Figure 8: Functional enrichment of differentially regulated proteins.

Analysis based on protein families (Pfam) identified the CLIP protease, proteasome sub unit A and insect cuticle proteins being down-regulated (Table 5).

Table 5: Biological pathways down-regulated in *An. gambiae s. s* larvae after exposure to cadmium heavy metal.

Pathway	Pathway ID	Pathway description	Observed gene count	False discovery rate	Regulation
Pfam	PF00089	Trypsin	30	1.89E-03	down
Pfam	PF00227	Proteasome subunit	7	1.89E-03	down
Pfam	PF10584	Proteasome subunit A N-terminal signature	5	3.37E-03	down
Pfam	PF12032	Regulatory CLIP domain of proteinases	7	7.71E-03	down
Pfam	PF00379	Insect cuticle protein	20	1.44E-02	down
Pfam	PF01105	emp24/gp25 L/p24 family/GOLD	4	2.78E-02	down
KEGG	190	Oxidative phosphorylation	21	2.00E-08	down
KEGG	1100	Metabolic pathways	58	3.32E-05	down
KEGG	910	Nitrogen metabolism	6	1.63E-04	down
KEGG	3050	Proteasome	9	7.43E-04	down
KEGG	1200	Carbon metabolism	12	1.72E-03	down
KEGG	10	Glycolysis / Gluconeogenesis	7	2.57E-03	down
KEGG	1120	Microbial metabolism in diverse environments	14	2.57E-03	down
KEGG	4145	Phagosome	9	2.57E-03	down
KEGG	1230	Biosynthesis of amino acids	9	2.77E-03	down
KEGG	3013	RNA transport	14	5.22E-03	down
KEGG	330	Arginine and proline metabolism	7	6.79E-03	down
KEGG	640	Propanoate metabolism	5	7.76E-03	down
KEGG	280	Valine, leucine and isoleucine degradation	6	1.43E-02	down
KEGG	270	Cysteine and methionine metabolism	5	1.45E-02	down

4.4.2 Down regulated proteins in cadmium treated mosquito larvae

The down-regulated proteins were by far the largest group (45) and these were functionally associated with glucose and carbohydrate metabolism, metal binding and immunity (Table 4 and Appendix 2). The most down-regulated was AGAPOO5163-PA, a UDP-glucuronosyl/UDP-glucosyltransferase. From the immune group were proteins from the Class II-associated invariant chain peptide (CLIP) family (AGAP000315-PA, AGAP004719-PA, AGAP009216-PA and AGAP010731-PA, AGAP011783-PA), prophenoloxidase (AGAP010730-PA) and GNBPl (AGAP004455-PA). Other proteins down-regulated were cuticular proteins (CPLCP13, CPTC1), UDP-galactose-4-epimerase, enzymes associated with redox pathway and free radical detoxification (AGAP004378 and AGAP005749), a protein involved with proteolysis (AGAP004015-PA), a ribosomal protein associated with oogenesis (RS3A_ANOGA), a cell growth regulator protein (AGAP006469-PA) and proton transport molecules.

4.4.3 Proteins induced by cadmium exposure in *An. gambiae s. s.* larvae

There was an induction of nine proteins with catalytic activity in the cadmium tolerant strain (Fig. 7 and Table 4). Three of these proteins were functionally associated with the redox pathway (AGAP010404-PB, AGAP001325 and AGAP002170) and actin dynamics (AGAP012056-PA). Two signaling molecules of the small GTPase family (AGAP001902 and AGAP002219), and several hypothetical proteins including two most abundant proteins (AGAP028204-PA and AGAP028068-PA) were up-regulated. Similarly, there was an induction of transcription factors, ribosomal proteins, enzyme from glutathione S transferase family (AGAP010404-PB) and proteins that function in the degradation pathway. Alpha tubulin (AGAP001219), previously demonstrated to be up-regulated in response to cadmium tolerance was also among the proteins that were found to be up-regulated.

CHAPTER FIVE

DISCUSSION

5.1 *An. gambiae s. s* colony establishment and maintenance

This study led to the establishment of a colony of cadmium heavy metal tolerant population and a control untreated population *An. gambiae s. s* strain. This strain can be used for further research work as stated in the recommendations or any heavy metal interrogative studies in *An. gambiae s. s* mosquitoes.

5.2 RNA Quantification

The presence of RNases in the environment makes RNA vulnerable to degradation. For downstream applications, high-quality RNA is needed, so RNA must be quantified to ensure its integrity. The ratio of RNA Absorbance to the Absorbance of the contaminants is used to estimate RNA purity. A260/A280 ratios of 1.8-2.2 are suitable for purity. From the RNA Quantification data, it was deduced that the extractions using the ZR Insect and Tissue RNA kit™ (Zymo Research Corporation, Irvine, CA, U.S.A.) yielded high quantities of RNA as compared to the use of the Trizol reagent (Invitrogen). However, it is quite clear that the quality of the RNA was not compromised. The quality of the RNA obtained met the purity criteria and therefore the extracted RNAs were considered suitable for downstream processes.

5.3 Differential transcript profiles in *An. gambiae s. s*

From the BLAST searches, over 70% of the identified genes were up-regulated as a result of cadmium heavy metal exposure while the rest were down regulated. The up-regulated genes were clustered into three biological functions which encompassed metabolism (AGAP008584, AGAP001249 and AGAP009563), transport (AGAP012302 and AGAP002638) and protein synthesis (AGAP028915, AGAP004750, AGAP028391, AGAP003870, AGAP028907, AGAP02818 and AGAP028899).

The ATP-binding cassette (ABC) transporters discovered in this study belong to the super family of membrane proteins that are present in all living organisms, including humans (Dean & Annilo, 2005). Inorganic ions, sugars, lipids lipopolysaccharides, metals, xenobiotics and drugs are all transported by ABC transporters across biological membranes (Dawson & Locher, 2006:

Hollenstein et al., 2007). Previous research on insects has found that since ABC transporters are involved in the movement of various substances across membranes, biological functions such as metabolism are harmed, especially when important components of metabolic reactions are transported and thus unavailable for efficient metabolic reactions in the organism. Production and insecticide tolerance are two other biological roles that have been harmed (Borycz et al., 2008; Dow & Davies, 2006; Ricardo & Lehmann, 2009; Vache et al., 2007). In addition, silencing an ABCH1 gene causes insects to die in their juvenile stages (Guo et al., 2015). The upregulation of these genes indicates that they are involved in cadmium transport across membranes for excretion, which reduces cadmium metal toxicity and thus aids larvae survival in the heavy metal environment. The up-regulation of the eupolytin gene may have a role in the activation of defense molecules in the mosquito larvae. The eupoltin-1 gene, which encodes a protease, was found to hydrolyze fibrinogen and activate plasminogen in a study involving the ground beetle *Eupolyphaga sinensis* and these defense molecules play a role in the bettle's immunity response (Yang et al., 2011). Ribosomal genes are involved in protein synthesis and the up-regulation of ribosomal RNAs in this study indicates that they play a role in enhancing *An. gambiae* survival in a heavy metal contaminated environment through transcription and translation of genes involved in the larvae's adaptation to heavy metals. In all species, the THO complex, which is a strongly conserved enzyme, is primarily responsible for maintaining proper gene expression and stability (Gewartowski et al., 2012; Köhler & Hurt, 2007). The THO complex's expression in this study suggests that it plays a role in expressing defense molecules that help larvae survive in a cadmium-polluted environment.

The down-regulated genes in this study were protein G12, endoplasmic reticulum metallopeptidase 1 and adenylate cyclase 8. G-proteins are surface receptors that couple receptors to adenylyl cyclase, controlling its activities such as raising cAMP levels and other events that make up the signal transduction pathway, which is essential for enhancing cellular responses. Endopeptidases and exopeptidases are enzymes that catalyze different activities in the cell, but all of their catalytic activities require a co-factor in the form of a metallic ion, which was discovered to be down-regulated in this research (Rawlings & Salvesen, 2013). The downregulation of these essential genes involved in signal transduction and proteolytic activity could explain the high rates of larval mortality seen in larvae raised in cadmium heavy metal environments. Through differential expression of complex transcripts, the data obtained in this

study sheds light on the transcriptional basis of *An. gambiae s. s* mosquito adaptation to cadmium metal. The expression of metallothionein in insects has been identified as a candidate gene for use as a biomarker for environmental pollution assessment (Hare, 1992; Klerks & Weis, 1987; Roesijadi, 1994). Alpha-tubulin has also been indicated as a heavy metal responsive gene in insects (Mattingly et al., 2001). Metallothionein was tested as a metal sensitive gene for Cd, Zn and Cu heavy metals using *Culex quinquefasciatus* mosquito larvae (Sarkar et al., 2004). As a result, the differentially expressed genes discovered in this research may be used to establish biomarkers to determine environmental stress or pollution in *An. gambiae s. s* mosquitoes.

5.4 Differential protein profiles in *An. gambiae s. s*

Previous studies had shown that cadmium tolerance leads to increased expression of alpha tubulin and metallothionein transcripts (Mireji et al., 2010a). This study has expanded the understanding on cadmium tolerance in mosquito by using an unbiased and more sensitive approach of combining the strength of 1-dimensional gel and a sensitive mass spectrometry analysis. From the proteomics work carried out in this study, 322 proteins have been identified to be differentially expressed in both the cadmium tolerant populations and the control populations. This was a marked improvement in the number of proteins established in a study undertaken previously (Mireji et al., 2006).

Results from this work indicate that there was a down regulation of various proteins and pathways that relate to immunity and stress responses in *Anopheles gambiae s. s* larvae. The relationship between heavy metal tolerance and mosquito immunity has previously not been well established. In this study, several immune molecules were down-regulated in cadmium exposed larvae. The importance of CLIPB9 and CLIPA8 for the activation of prophenoloxidase cascade and melanization of *Plasmodium* and bacteria has been demonstrated (An et al., 2011). GNBP1 (AGAP004455-PA) plays a vital role by acting as a pattern recognition receptor (PRR) with a broad repertoire for pathogen surveillance (Dimopoulos, 2003; Whitten et al., 2004). The TOLL and IMD pathways are activated by the pattern recognition receptor, causing mosquito immunity genes to be expressed. In the absence of effective immune surveillance, heavy metal tolerant mosquitoes may be more prone to bacterial pathogens frequently found in aquatic habitats. Also, immune priming occurs at larval stages and shapes anti-plasmodia responses in adult stages (Moreno-Garcia et al., 2015). However, the immune system development is a complex process

and may be different in laboratory settings as compared to a natural environment where other abiotic factors may come into play. Other immune molecules in this cluster were SRPN11, SRPN12, APOII, CTL4, CTL8, GALE 8 and LRIM26 (Appendix 2). Serpins (AGAP001375-PA and AGAP001377-PA) are irreversible inhibitors of serine proteases which are involved in a wide range of physiological and pathological reactions in humans and insects especially innate immune responses (Janciauskiene, 2001). Insect genomes have been shown to contain serpin genes and they are thought to protect insect from pathogen attack by inhibiting proteases produced by fungi and parasites among other functions (Kanost et al., 1990; Zou et al., 2009). The down regulation of serpin genes observed in this study supports the reduced immunity in the larvae due to heavy metal exposure and hence the high mortality rates of larvae (Mireji et al., 2010b). The down regulation of these proteins would also point to the reduction of the larvae numbers previously reported due to lack of proper body mechanisms of encountering the sting of heavy metal pollution (Mireji et al., 2010b). Heat-shock proteins (HSP) were also found to be down regulated (AGAP002076-PA, AGAP004192-PA, AGAP001424-PA) in this study. Insects have been discovered to have high levels of heat shock proteins, which function as survival modulators. They are a group of functionally linked proteins that help other proteins fold and unfold. In response to environmental inputs such as heat shock, ultraviolet radiation, chemical pesticides and biotic stress such as viruses, bacteria, fungi and other insects, the expression of different HSP genes is induced and modulated in insects (Zhao & Jones, 2012). In the sleeping chironomid *Polypedilum vanderplanki*, heat shock proteins were discovered to be critical up-regulated genes for anhydrobiosis (Cornette et al., 2010). Heat shock proteins (HSP70, HSP90) were the most sensitive to changes in hydration state in all genes examined in a study to determine the molecular responses of dehydration, rehydration and over hydration in larvae of the Antarctic midge *B. Antarctica* (Lopez-Martinez et al., 2009*).* HSPs are caused by viruses, bacteria, fungi and insects to provide defense against stressors, according to numerous studies (Hong et al., 2010; Lyupina et al., 2010*;* Rungrassamee et al., 2010; Ying & Feng, 2011). Results from this research work demonstrate a down regulation of HSP70 and HSP90 and this acts as an indicator of reduced larvae numbers that are usually observed during the transition from one instar stage to the next in the cadmium treated larvae.

Results from this study demonstrated a down regulation of the entire signal transduction mechanism. Signaling mechanisms are crucial not only for general cellular processes but also for

sensing danger in form of pathogen infection. The mosquito deploys several strategies to prevent or respond to invading pathogens. The insect exoskeleton which is largely composed of chitin provides a physical barrier against invading pathogens, with the second level of barrier that targets pathogens that have made it through into the system being provided by antimicrobial peptides, immune genes and hemocytes. Low expression of cuticular proteins was observed in this study suggesting reduced capacity to respond to heavy metals and a compromised exoskeleton which can partly explain the reduced survival rates in larvae, pupa and adults emergence as previously reported (Mireji et al., 2010b).

There was a down regulation of genes that decode enzymes involved in the metabolism of carbohydrates. The most significant change observed in this study was that of AGAP005163-PA, a UDP-glucuronosy1/UDP-glucosyltransferase that was consistently not identified in heavy metal tolerant mosquitoes. This was not surprising given that UDP-glucuronosyl catalyzes the transfer of glucose from UDP-glucose to ecdysteroids and the vital role ecdysteroids play in regulating insect growth and transitions between stages. Low levels of ecdysteroids are linked to a disruption in mosquito development. Heavy metal tolerance is known to induce delays in pupation and reduced adult size. Thus, AGAP005163-PA abundance can be associated with the fitness cost incurred for heavy metal adaptation. Other down-regulated enzymes in this study included, Pyrophosphatase, Lactate/malate Dehydrogenase and Triose Phosphate Isomerase among others (Appendix 2). These metabolic enzymes are necessary for glycolysis, which is required for efficient energy production (Jung et al., 2002). Interestingly, vulnerability to pathogen infections is a feature of triose phosphate isomerase deficiency, a rare glycolytic enzymopathy that affects humans and other mammals (Schneider, 2000). The low expression of metabolic enzymes observed in this study would translate to inefficient energy production required for normal growth and development thereby attributing to the high mortality rates observed in cadmium exposed larvae.

Proteasome protein was found to be down regulated in the larvae after cadmium exposure. The proteasome is involved in both the ATP-dependent degradation of naturally occurring unstable regulatory proteins and the uncontrolled destruction of constitutively long-lived proteins in the non-lysosomal pathway. Furthermore, it appears to be essential for maintaining cell viability to rapidly remove proteins with abnormal structures that may be produced in cells as a result of mutations or various environmental stresses such as oxidation and

heavy metal exposure, implying that proteolysis may act as a self-protective mechanism to protect cells from such stresses (Tanaka, 1998). The Ubiquinone-proteasome pathway is responsible for the selective degradation of certain harmful proteins. Actually, the poly-ubiquinone gene, which belongs to the heat shock gene family, encodes ubiquinone, implying that under stress, cells must generate a large number of ubiquinone molecules to promote the rapid degradation of abnormal proteins caused by the stress (Hochstrasser, 1997; Varshavsky, 1997). Furthermore, the ribosomal protein RS3A (AGAP003532-PA), which has been shown to play a role in exogenesis, was down regulated, which is consistent with the observed decrease in fecundity.

Although there was a general down-regulation of the oxidative phosphorylation pathway in this study, it was observed that enzymes from the GST family were significantly up-regulated. GSTs function by catalyzing the conjugation of glutathione (GSH) (Xu et al., 2014) to various electrophonic compounds, including reactive oxygen species (ROS) These molecules are induced as a result of oxidative stress caused by the accumulation of reactive oxygen species (Li et al., 2003). Deleterious effects of oxidative stress are not only associated with tissue, DNA and protein damage, but reports indicate this may include insecticides resistance mechanisms in mosquitoes (Jones et al., 2012; Oliver & Brooke, 2018; Ramirez & Gimenez, 2003). High levels of metabolic enzymes such as GSTs and cytochrome oxidase are strongly associated with this phenotype (Oliver & Brooke, 2016). As insects become more accustomed to heavy metal polluted habitats, there is an excellent potential for increased risk of high-level insecticides resistance and increased odds of disease transmission. There was an up regulation of nine proteins with catalytic activity in the cadmium stressed larvae (Appendix 3). Three of these serve in the redox pathway (AGAP001711, AGAP001325 and AGAP002170) and three are involved in hydrolysis (AGAP000862, AGAP000573 and AGAP002055). Two signaling molecules of the small GTPase family (AGAP001902 and AGAP002219) were also up-regulated. It was also observed that alpha tubulin (AGAP001219) that had previously been demonstrated to be up-regulated in response to cadmium tolerance was among the proteins reported in this group (Mireji et al., 2010a). Other proteins induced included transcription factor, ribosomal proteins and those in the protein degradation pathway involving ubiquitination. The induction of these proteins is a response to cadmium heavy metal and they are responsible for the observed adaptation of the *Anopheles gambiae s. s* larvae to heavy metal polluted environment. However,

the main limitations for this study includes 1) a single focus on *An. gambiae s. s.* among other Afro-tropical mosquito vectors of malaria, 2) limitation to cadmium tolerance among other heavy that are also present in *An. gambiae* habitats (Mireji et al., 2008) and 3) the study only focused on one developmental stage (larvae) and yet all other developmental stages are important in mosquito survivorship and associated vectorial capacity.

CHAPTER SIX

CONCLUSIONS AND RECOMMMENDATIONS

6.1 Conclusions

Data presented in this study illustrated the use of Trizol reagent and ZR Tissue and Insect RNA Microprep kit (Zymo Research Corporation, Irvine, CA, U.S.A.). The ZR Tissue and Insect RNA Microprep kit generated a higher quantity of extracted RNA as compared to the use of Trizol reagent. However, the RNA generated in both cases was of similar quality and was considered suitable for all down stream processes. In addition, the data suggests that there are tradeoffs between cadmium tolerance and insect capacity to undergo normal development and mount an effective immune response to cadmium metal.

This study has advanced the existing field of knowledge in the following ways:-

i. *An. gambiae s. s* established colony has the potential capacity to survive and reproduce in a cadmium heavy metal polluted environment.
ii. *An. gambiae s. s* mosquitoes express repertoire of genes in response to cadmium heavy metal tolerance.
iii. *An. gambiae s. s* mosquitoes express protein profiles specific to cadmium heavy metal exposure.

6.2 Recommendations

Based on the results obtained from this study, the following recommendations are made;

i. Further work should be carried out with *An. gambiae s. s* larvae from naturally polluted environments to find out how the larvae in these settings compare with those in laboratory settings and also for validation of the present findings.
ii. The function of differentially expressed genes (DEGs) in *An. gambiae s. s* health and reproductive success should be investigated further.
iii. The current study identified AGAP005163-PA candidate protein that deserves further interrogation for potential application in development of biomarkers for assessment of cadmium heavy metal environmental contamination.

REFERENCES

Abdalla, C., Dumas-Gaudot, E., Renaut, J., & Sergeant, K. (2012). Gel-based and gel-free quantitative proteomics approaches at a glance. *International Journal of Plant Genomics, 2012,* 1-17.

Aebersold, R., & Mann, M. (2003). Mass spectrometry-based proteomics. *Nature*, *422*, 198-207.

Aebersold, R., & Mann, M. (2016). Mass spectrometric exploration of proteome structure and function. *Nature, 537,* 347-355.

Afify, A., Betz, J. F., Riabinina, O., Lahonde, C., & Potter, C. J. (2019). *Commonly used Insect Repellents.* Handbook, Second Edition (CRC Press).

Afrane, Y. A., Klinkenberg, E., Drechsel P., Owusu- Daaku, K. Garms, R., & Kruppa, T. (2004). Does irrigated urban agriculture influence the transmission of malaria in the city of Kumasi, Ghana? *Acta Tropica, 89,* 125-134.

Agaba, T. A., Fawole, B., & Claudius-Cole, B. (2015). Screening of Siam Weed (*Chromolaena o**dorata***) and African custard Apple (*Annona senegalensis*) for Nematicidal Activity. *Journal of Biology Agriculture and Heathcare, 5*(14), 50-56.

Ahnert, N., Patton, W., & schulenberg, B. (2004). Optimized conditions for diluting and reusing a fluorescent protein gel stain. *Electrophoresis, 25*(15), 2506-2510.

Aikawa, M. (1980). *Host cell invasion by malarial parasites. In cellular interactions in symbiosis and parasitism* (C. B. Cook, P. W. Pappas and E. D. Rudolph eds). Ohio University Press, Colombus, USA, 31-46.

Aikawa, M., & Seed, T. M. (1980). *Morphology of Plasmodia in: Malaria* (J.P Kreier ed.). Academic Press, New York. 1, 285-344.

Alonso, P. L., Lindsay, S. W., Armstrong, J. R. M., Conteh, M., Hill, A. G., Fegan, G., De Francisco, A., Hall, A. J., Shenton, F. C., Cham, K., & Greenwood, B. M. (1991). The effect of insecticides treated bed nets on mortality of Gambian children. *Lancet, 337,* 1499-1502.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25* (17), 3389-3402.

Amster, I. J. (1996). A tutorial on Fourier Transform Mass Spectrometry. *Journal of Mass Spectrometry, 31,* 1325-1337.

An, C., Budd, A., Kanost, M. R., & Michel, K. (2011). Characterization of a regulatory unit that controls melanization and affects longevity of mosquitoes. *Cell and Molecular Life Sciences, 68*, 1929-1939.

Anderson, N., & Anderson, N. (1996). Twenty years of two-dimensional electrophoresis: past, present and future. *Electrophoresis, 17*(3), 443-453.

Andrews, G. L., Simons, B. L., Young, J. B., Hawkridge, A. M., & Muddiman, D. C. (2011). Performance characteristics of a new hybrid quadrupole time-of-flight tandem mass spectrometer (TripleTOF5600). *Analytical Chemistry, 83*(13), 5442-5446.

Angotti, T. (1993). *Metropolis 2000*: Planning, Poverty and Politics. London, Routledge.

Armengol, G., Hernandez, J., Velez, J. G., & Orduz, S. (2006). Long-lasting effects of a *Bacillus thuringiensis* serovar *israelensis* experimental tablet formulation for *Aedes aegypti* (Diptera: Culicidae) control. *Journal of Ecotoxicological Entomology, 99,* 1590-1595.

Atafar, Z., Mesdaghinia, A., & Nouri, J. (2010). Effect of fertilizer application on soil heavy metal concentration. *Environmental Monitoring and Assessment, 160,* 83-89.

Atyame, C. M., Labbé, P., Lebon, C., Weill, M., Moretti, R., Marini, F., Gouagna, L. C., Calvitti, M., & Tortosa, P. (2016). Comparison of irradiation and *Wolbachia* based approaches for sterile male strategies targeting *Aedes albopictus*. *PloS ONE 11*(1), e0146834.

Augustyniak, M., Babczynska, A., Migula, P., Wilczek, G., Laszczyca, P., Kafel, A., & Augustyniak, M. (2005). Joint effects of dimethoate and heavy metals on metabolic responses in a grasshopper (*Chorthippus brunneus*) from a heavy metals pollution gradient. *Comparative Biochemistry and Physiology, 141,* 412-419.

Augustyniak, M., Babczynska, A., & Augustyniak, M. (2009). Does the grasshopper *Chorthippus brunneus* adapt to metal polluted habitats? A study of glutathione-dependent enzymes in grasshopper nymphs. *Insect Science, 16,* 33-42.

Awolola, T. S., Oduola, A., Obansa, J. B., Chukwurar, N. J., & Unyimandu, J. P. (2007). *Anopheles gambiae s. s* breeding in polluted water bodies in urban Lagos, southwestern Nigeria. *Vector Borne Diseases, 44,* 241-244.

Ayeni, K., Ajayib, O., & Odeyemic, D. (2017). Studies on heavy metal accumulation in plant, insect and soil in a public dumpsite in Ado-Ekiti, Ekiti State. *International Journal of Sciences*: *Basic and Applied Research, 36*(2), 18-26.

Azam, S., Afsheen, A., Zia, M., Javed, R., Saeed, M., Sarwar, B., & Munir, M. (2015). "Evaluating insects as bioindicators of heavy metal contamination and accumulation near Industrial area of Gujrat, Pakistan". *BioMedical Research International, l,* 11-13.

Bachem, C. W., van der Hoeven, R. S., & de Bruijin, S. M. (1996). Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. *Plant Journal, 9* (5), 745-753.

Bai, B., & Laiho, M. (2012). Efficient sequential recovery of nucleolar macromolecular components. *Proteomics, 12,* 3044-3048.

Bailey, P. C. E. (1989). The effect of water temperature on the functional-response of water stick insect *Ranatra dispar* (Heteroptera: Nepidae). *Australian Journal of Ecology, 14,* 381-386.

Baldwin, M. A. (2005). Mass spectrometers for the analysis of biomolecules. *Methods in Enzymology, 402,* 3-48.

Bantscheff, M., Dumplefield, B., & Kuster, B. (2004). Femtomol sensistivity post digest ^{18}O labeling for relative quantification of differential protein complex composition. *Rapid Communication in Mass Spectrometry, 18* (8), 869-876.

Bayoh, M. N., & Lindsay, S. W. (2004). Temperature-related duration of aquatic stages of the Afrotropical malaria vector mosquito *Anopheles gambiae* in the laboratory. *Medical Veterinary Entomology, 18,* 174-179.

Beck, S., Michalski, A., Raether, O., Lubeck, M., Kaspar, S., Goedecke, N., & Mann, M. (2015). The impact II, a very High-Resolution Quadrupole Time-of-Flifgt Instrument (Q-TOF) for Deep Shotgun proteomics. *Molecular and Cellular Proteomics, 14*(7), 2014-2029.

Becker, A., Chao, D-Y., Zhang, X., Salt, D. E., & Baxter, I. (2011). Bulk Segregant Analysis Using Nucleotide Polymorphism Microarrays. *PLoS ONE, 6*(1), e15993.

Beier, J. C., Oster, C. N., Onyango, F.K., Bales, D. J., Sharwood, J. A., Perkins, P. V, Chumo, D. K., Koech, D. K., Whitemire, R. E., Diggs, C. L., & Hoffman, S. L. (1994). *Plasmodium falciparum* incidences relates to entomologoe inoculation rates at a site proposed for testing malaria vaccines in Western Kenya. *American Journal of Tropical Medicine and Hygiene, 50,* 529- 536.

Bellini, R., Balestrino, F., Medici, A., Gentile, G., Veronesi, R., & Carrieri, M. (2013a) Mating competitiveness of *Aedes albopictus* radio-sterilized males in large enclosures exposed to natural conditions. *Journal of Medical Entomology, 50,* 94-102.

Bellini, R., Medici, A., Puggioli, A., Balestrino, F., & Carrieri, M. (2013b). Pilot field trials with *Aedes albopictus* irradiated sterile males in Italian urban areas. *Journal of Medical Entomology, 50,* 317-325.

Benedict, M. Q. (1997). *Care and maintenance of Anopheline mosquito colonies.* In: Crampton JM, Beard CB, Louis C (eds). The Molecular Biology of Insect Disease Vectors. Chapman & Hall, New York, pp 2-12

Benedict, M. Q., & Robinson, A. S. (2003). The first release of transgenic mosquitoes: An argument for the sterile insect technique. *Trends in Parasitology, 19,* 349-355.

Benelli, G., Jeffries, C. L., & Walker, T. (2016). Review of Biological Control of Mosquito Vectors: past, present and future. *Insects Review, 7*(4), 52

Benson, L. M., Null, A. P., & Muddiman, D. C. (2003). Advantages of *thermococcus kodakaraenis* (KOD) DNA polymerase for PCR-mass spectrometry based analyses. *Journal of American Society of Mass Spectrometry, 14,* 601-604.

Beyersmann, D., & Hechtenberg, S. (1997). Cadmium, gene regulation and cellular signaling in mammalian cells. *Toxicology & Applied Pharmacology, 144,* 247-261.

Bhattarai, A. (2007). Impact of artemisinnin based combination therapy and insecticide-treated nets on malaria burden in Zanzibar; *PloS Medicine 4,* 309-310.

Bier, F. F., Nickisch-Rosenegk, M., Ehrentreich-Förster, E., Reib, E., Henkel, J., Strehlow, R., & Andresen, D. (2008). DNA microarrays. *Advances in Biochemical Engineering/ Biotechnology, 109,* 433-453.

Billy, T. F., Edmond, K., Cyrille, N., Benjamin, M. D., Carlo, C., Flaubert, N., Parfat, A. A., & Christopher, A. N. (2012). Water Quantity and *Anophelies gambiae* larval tuberance to Pyrethreoids in the Cities of Doula and Yaound'e (Cameroon). *Journal of Tropical Medicine, 2012,* Article ID 429817.

Biney, C., Amaza, A. T., Calamari, D., Kaba, N., Mbome, I. L., Naeve, H., Ochumba, P. B. O., Osibanjo, O., Radegonde, V., & Saad M. A. H. (1994). Review of heavy metals in Africa aquatic environment. *Ecotoxicological Environmental Safety, 28,* 134-159.

Bjellqvist, B., E. K., Righetti, P., Gianazza, E., Gorg, A., Westermeier, R., & Postel, W. (1982). Isolectric focusing in immobilized pH gradients: Principle, methodology and some applications. *Journal of Biochemical and Biophysical Methods, 6*(4), 317-339.

Bockarie, M. J., Service, M. W., Barnish, G., Momoh, W., & Salia, F. (1994). The effect of woodsmoke on the feeding and resting behavior of *Anopheles gambiae s. s. Acta Tropica, 57,* 337-340

Bodzon-kulakowska, A., Bierczynska-Krzysik, A., Dylag, T., Drabik, A., Suder, P., Noga, M., Jarzebinska, J., & Silberring, J. (2007). Methods for samples preparation in proteomic research. *Journal of Chromatography B, 849,* 1-31.

Bondy, S. C. (2016). *"Metal toxicity, inflammation and oxidative stess" in inflammation, Aging and Oxidative stress Oxidative Stress in Applied Basic Research and Clinical Practice,* eds S. Bondy, A. Campbell (Cham: Springer International Publishing), 3-16.

Borycz, J., Borycz, J. A., Kubow, A., & Llyod, V. (2008). *Drosophila* ABC transporter mutants white brown and scarlet have altered contents and distribution of biogenic amines in the brain. *Journal of Experimental Biology, 211,* 3454-3466.

Bossio, R. E., & Marshall, A. G. (2002). Baseline resolution of isobaric phosphorylated and sulfated peptides and nucletides by electrospray ionization FTICR MS: Another step toward mass spectrometry-based proteomics. *Analytical Chemistry, 74,* 1674-1679.

Bowatte, G., Perera, P., Senevirathne, G., Meegaskumbura, S., & Meegaskumbura, M. (2013). Tadpoles as dengue mosquito (*Aedes Aegypti*) egg predators. *Biological Control, 67,* 469-474.

Bozdech, Z., Llinas, M., Pulliam, B. L., Wong, E, D., Zhu, J., & DeRisi, J. L. (2003). The transcriptome of the intra-erythrocytic development cycle of *Plasmodium falciparum. Public Library of Science in Biology, 1,* 85-100.

Brabin, B. J. (1983). An analysis of malaria in pregnancy in Africa. *Bulletin of World Health Organization, 61,* 1005-1016.

Bradford, M. M. (1976). A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry, 72,* 248-254.

Breackmann, B., Brys, K., Rzeznik, U., & Raes, H. (1999). Cadmium pathology in an insect cell line: ultrastructural and biochemical effects. *Tissue &Cell, 31,* 45-53.

Breman, J. G., Alilio, M. S., & White, N. J. (2007). Defining and defeating the intolerable burden of marlaria III. Progress and perspectives. *American Journal of Tropical Medicine and Hygiene, 77,* vix–xi.

Brodman, R., Dorton, R. (2006). The effectiveness of pond-breeding salamanders as agents of larval mosquito control. *Journal of Freshwater Ecology, 21,* 467-474.

Bronstrup, M. (2004). Absolute quantification strategies in proteomics based on mass spectrometry. *Expert Review of Proteomics, 1*(4), 503-512.

Brooke, B., Kioke, G., & Hunt, R. (2001). Bioassay and Biochemical analyses of insecticide resistance in African *Anopheles funestus* (Diptera: Culicidae). *Bulletin of Entomological Research, 91,* 265-272.

Brown, M., & Hebert, A. A. (1997). Insect repellents: An Overview. *Journal of American Academy of Dermatology, 36,* 243-249.

Budiansky, S. (2002). Creatures of our own making. *Science, 298,* 80-86.

Burt, P. E., & Goodchild, R. E. (1974). Knockdown by pyrethroids: Its role in the intoxication process. *Pesticide Science, 5,* 625-633.

Caraballo, H., & King, K. (2014). Emergency department management of mosquito-borne illness: Malaria, dengue and west Nile virus. *Emergency Medicine Practice, 16* (5), 1-23.

Carlsen, S. C. & Fomsgaard, I. S. (2008). Biologically active secondary metabolites in white clover (*Trifolium repens L.).* A review focusing on contents in the plant, plant-pest interactions and transformations. *Chemoecology, 18,* 129-170.

Carrascal, M., Ovelleiro, D., Casas, V., Gay, M., & Aban, J. (2008). Phosphorylation Analysis of Primary Human T Lymphocytes Using Sequential IMAC and Titanium Oxide Enrichment. *Journal of Proteome Research, 7*(12), 5167-1576.

Carter, R., & Mendis, K. N. (2002). Evolutionary and historical aspects of the burden of malaria. *Clinical Microbiology Review, 15,* 564-594.

Casneuf, T., van de Peer, Y., & Huber, W. (2007). *In situ* analysis of cross-hybridisation on microarrays and the inference of expression correlation. *BMC Bionformatics, 8,* 461.

Cecchini, E., Dominy, P. J., Geri, C., Kaiser, K., Sentry, J., & Milner, J. J (1993). Identification of genes up-regulated in differentiating *Nicotania galuca* pith tissue, using an improved method for constructing a subtractive cDNA library. *Nucleic Acids Research, 21,* 5742-5747.

Cha, B., Blades, M. W., & Douglas D. J. (2000). An interface with a Linear Quadruple Ion Guide for an Electrospray-Ion Trap Mass Spectrometer System. *Analytical Chemistry, 72*, 5647-5654.

Champer, J., Buchman, A., & Akbari, O. S. (2016). Cheating evolution: Engineering gene drives to manipulate the fate of wild populations. *Nature Review of Genetics, 17*, 146-159.

Chandra, G., Bhattacharjee, I., Chatterje, S. N., & Ghosh, A. (2008). Mosquito control by larvivorous fish. *Indian Journal of Medical Research, 127*, 13-27.

Chandre, F., Barrier, F., & Manga, L. (1999). Status of pyrethroid resistance in *Anopheles gambiae sensu lato. Bulletin of the World Health Organization, 77,* 230-234.

Chang, H. C., Hsu, F., Freeman, G. J., Griffin, J. D., & Reinherz E. L. (1989). Cloning and expression of a gamma-interferon-inducible gene in monocytes: A new member of a cytokine gene family. *International Immunology, 1,* 388-397.

Charlese, F. B., Sullivan, W. W., & Smith, L. W. (1995). Use and status of DDT in Zimbabwe. *Journal of African Health Science, 2,* 11-69.

Chaurand, P., Luetzenkirchen, F., & Spengler, B. (1999). Peptide and protein identification by matrix-assisted laser desorption ionization (MALDI) and MALDI post-source decay time of-flight mass spectrometry. *Journal of the American Society for Mass Spectrometry, 10*(2), 91-103.

Chen-Hussey, V., Carneiro, L., Keomanila H., Gray R., Bannavong S., Phanalasy, S., & Lindsay, S. W. (2013). Can topical insect repellents reduce malaria? A cluster-randomised controlled trial of the insect repellent N, N-diethyl-m-toluamide (DEET) in Lao PDR. *PLoS ONE, 8*(8), e70664.

Chien, K. Y., Liu, H. C., & Goshe, M. B. (2011). Development and Application of phosphoproteomic method using Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC), IMAC and LC-MS=MS Analysis to study Mareks's Disease Virus Infection. *Journal of Proteome Research, 10*(9), 4041-4053.

Chinery, W. A. (1984). Effects of ecological changes on the malaria vectors *Anopheles funestus* and *Anopheles gambiae complex* of mosquitoes in Accra, Ghana. *Journal of Tropical Medicine and Hygiene, 87,* 75-81.

Chinery, W. A. (1995). Impact of rapid urbanization on mosquitoes and their disease transmission potential in Accra and Tema, Ghana. *African Journal of Medicine and Science, 24*, 179-188.

Chitra, T., Murugan, K., Naresh Kumar, A., Madhiyazhagan, P., Nataraj, T., Indumathi, D., & Hwang, J. S. (2013). Laboratory and field efficacy of *Pedalium murex* and predatory copepod *Mesocyclops longisestus* on rural malaria vector *Anopheles Culicifacies*. *Asian Pacific Journal of Tropical Diseases, 3,* 111-118.

Chobu, M., Nkwengulila, G., Mahande, A. M., Mwang'onde, B. J., & Kweka, E. J. (2015). Direct and indirect effect of predators on *Anopheles gambiae sensu stricto. Acta Tropica, 142*, 131-137.

Choe, L., D'Ascenzo, M., Relkin, N. R., Pappin, D., Ross, P., Williamson, B., Guertin, S., Pribil, P., & Lee, K. H. (2007). 8-plex quantitation of changes in cerebrospinal fluid protein expression in subjects undergoing intravenous immunoglobulin treatment for Alzheimer's disease. *Proteomics, 7,* 3651-3660.

Christov, V., Simeonov, M., Velcheva, N., Karadjova, O., Atannassov, N., Ivanova, I., & Evstatieva, L. (1997). Pyrrolizidine Alkaloids from Bulgarian Species-genus Senecio and their insecticidal properties. *Biotechnology and Biotechnological equipment, 11*(1-2), 53-59.

Claassen, M., Reiter, L., Hengartner, M. O., Buhmann, J. M., & Aebersold, R. (2012). Generic comparison of protein inference engines. *Molecular and Cellular Proteomics, 11*(4), 0110.007088.

Clements, W. H., & Kifney, R. M. (1994). Integrated laboratory and field approach for assessing impact of heavy metals at the Arkansas River, Colorado. *Environmental Toxicology and Chemistry, 7,* 715-722.

Cloarec, A. (1990). Factors influencing the choice of predatory tactics in a water bug, *Diploychus indicus* Venk and Rao (Heteroptera, Belostomatidae). *Journal of Animal Behaviour, 40,* 262-271.

Cloonan, N., Forrest, A. R., Kolle, G., Gardiner, B. B., Faulkner, G. J., Brown, M. K., Taylor, D. F., Steptoe, A. L., Wani, S., Bethel, G., Roberston, A. J., Perkins, A. C., Bruce, S. J., Lee, C. C., Ranade, S. S., Peckham, H. E., Manning, J. M., McKernana, K. J., & Grimmoond,

S. M. (2008). Stem cell transcriptome profiling via massive scale mRNA sequencing. *Nature Methods, 5,* 613-619.

Coene, J. (1993). Malaria in urban and rural Kinshasa: The entomological input. *Medical and Veterinary Entomology, 7,* 127-137.

Coetzee, M., Hunt, R. H., & Wilkerson R. A. (2013). *Anopheles coluzzii* and *Anopheles amharicus,* new members of the *Anopheles gambiae complex*. *Zootaxa,* 19(3), 246-274.

Coleman, R. E. L., Robert, L., Roberts, L. W., Glass, J. A., Seeley, D. C., Laughinghouse, A., Perkins, R. V., & Wirtz, R. A. (1993). Laboratory evaluation of repellents against four anopheline mosquitoes (Diptera: Culicidae) and two phlebotomine sandflies (Diptera: Psychodidae). *Journal of Medical Entomology, 30,* 499-502.

Collins, F. H., & Besansky, N. J. (1994). Vector biology and the control of malaria in Africa. *Science, 264,* 1874-1875.

Collins, F. H., & Paskewitz, S. M. (1995). Malaria: Current and future prospects for control. *Annual Review of Entomology, 40,* 195-219.

Coluzzi, M. (1984). Heterogeneites of the malaria vectorial system in tropical Africa and their significance in malaria epidemiology and control. *Bulletin of World Health Organization, 62,* 107-113.

Coluzzi, M., Petrarca, V., & Di Deco, M. A. (1985). Heterogeneities of the malaria vectorial system in tropical Africa and their significance in malaria prevention and control. *Bulletin of World Health Organization, 62,* 107-113.

Coluzzi, M. (1992). Malaria vector analysis and control. *Parasitology Today, 8,* 113-118.

Coluzzi, M. (1993). Advances in the study of Afrotropical malaria vectors. *Parasitology, 35,* 23-29.

Comisarow, M. B. & Marshall, A. G. (1974). Fourier Transform Ion Cyclotron Resonance Spectroscopy. *Chemical Physics Letters, 25,* 282-283.

Cooks, R. G. (1995). Collision-induced dissociation: Readings and commentary. *Journal of Mass spectrometry, 30,* 1215-1221.

Coraballo, H. & King, K. (2014). Emergency department management of mosquito-borne illness: malaria, dengue and West Nile virus. *Emergency Medicine Practice, 16*(5), 1-23.

Corbel, V., N'Guessan, R., Brengues, C., Chandre, F. & Djogbenou, L. (2007). Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. *Acta Tropica, 101,* 207-216.

Cornette, R., Kanamori, Y., Watanabe, M., Nakahara, Y., Gusev, O., & Mitsumasu, K. (2010). Identification of anhydrobiosis-related genes from an expressed sequence tag database in the cryptobiotic midge *Polypedilum vanderplanki* (Diptera: Chironomidae). *Journal of Biological Chemistry, 285,* 35889-35899,

Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology, 26,* 1367-1372.

Cox-Singh, J., Davis, T. M., Lee, K. S., Shamsul, S. S., Matusop, A., Ratnam, S., Rahman, H. A., Conway, D. J., & Singh, B. (2008). *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clinical Infectious Diseases, 46,* 165-171.

Cui, X. S., Shin, M. R., Lee, K. A., & Kim, N. H. (2005). Identification of differentially expressed genes in murine embryos at blastocyst stage using annealing control primer system. *Molecular and Reproductive Development, 70,* 278-287.

Curtis, C. F. (1992). Personal protection methods against vectors of disease. *Review of Medical Veterinary Entomolology, 80,* 543-553.

Dai, J., Shieh, C. H., Sheng, Q. H., Zhou, H., & Zeng, R. (2005). Proteomic analysis with integrated multiple dimensional liquid chromatography/mass spectrometry based on elution of ion exchange column using pH steps. *Analytical Chemistry, 77*(18), 5793-5799.

Das, M. K., & Prasad, R. N. (1973). Evaluation of mosquito fish *Gambusia affinis* in the control of mosquito breeding in rice fields. *Indian Journal of malaria, 28,* 171-177.

Davidson, G. (1962). *Anopheles gambiae* complex. *Nature, 196,* 907.

Davidson, G., & Hunt R. H. (1973). The crossing and chromosome characteristics of a new 6th species in the *Anopheles gambiae* complex. *Parasitologia, 15*(1-2), 121-128.

Davis, M. M., Cohen, D. I., Nielsen, E. A., Steinmetz, M., Paul, W. E., & Hood, L. (1984). Cell type-specific cDNA probes and the murine I region: the localization and orientation of

Ad alpha. *Proceedings of the National Academy of Science of the United States of America, 81,* 2194-2198.

Davis, J. M. (1991). Statistical theory of spot overlap in two-dimensional separations. *Analytical Chemistry, 63,* 2141-2152.

Dawson, P. H. (1986). Quadrupole mass analyzers: Perfomance, design and some recent applications. *Mass Spectrometry Review, 5,* 1-37.

Dawson, D. B. (1990).Use of nucleic acid probes in genetic tests. *Clinical Biochemistry, 23,* 279-285.

Dawson, R. J., & Locher, K. P. (2006). Structure of a bacterial multidrug ABC transporter. *Nature, 443,* 180-185.

de Bievre, P. & Taylor, P. D. P. (1993). Table of the isotopic composition and elements. *International Journal of Mass Spectrometry, 123,* 149-166.

de Boer, H., Vongsombatjh, C., Pålsson, K., Björk, L., & Jaenson, T. G. T. (2010). Botanical repellents and pesticides traditionally used against hematophagous invertebrates in Lao people's Democratic Republic: A comparative study of plants used in 66 villages. *Journal of Medical Entomology, 47*(3), 400-414.

de Castro, M. C., Monte-Mér, R. L., Sawyer, D. O., & Singer, E. H. (2006). Malaria risk on the Amazon Fontier. *Proceedings of the National Academy of Sciences of the United States of America, 103,* 2453-2457.

Dean, M., & Annilo, T. (2005). Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. *Annals Review of Genomics and Human Genetics, 6,* 123-142.

Debboun, M., Frances, S. P., & Strickman, D. (2006). *Insect Repellents: principles Methods, and Uses.* CRC Press

Debboun, M., Frances, S., & Strickman, D. (2014). *Insect repellents*. Handbook, second Edition (CRC Press).

Delmotte, N., Lasaosa, M., Tholey, A., Heinzle, E., & Huber, C. G. (2007). Two –dimensional reversed-phase × ion pair reversed-phase HPLC: An alternative approach to high-resolution peptide separation for shotgun proteome analysis. *Journal of Proteome Research, 6*(11), 4363-4373.

Desiderio D. M., & Kai M. (1983). Preparation of stable isotopeincorporated petide internal standards for field desorption mass spectrometry quantification of peptides in biological tissues. *Biomedical Mass Spectrometry, 10,* 471-479.

Deutsch, E.W., Csordas, A., Sun, Z., Jarnuczak, A., Perez-Riverol, Y., Ternent, T., Campbell, D.S., Bernal-Llinares, M., Okuda, S., & Kawano, S. (2017). The ProteomeXchange consortium in 2017: Supporting the cultural change in proteomics public data deposition. *Nucleic Acids Research, 45,* D1100-D1106.

Diabate`, A., Baldet, T., & Chandre, F. (2002). The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae s. 1.* in Burkina Faso. *American Journal of Tropical Medicine and Hygiene, 67,* 617-622.

Diabete` A., K. R., Dabire, E. H., Kim, R., Dalton. N., Millogo, T., Baldet, F., Simard, J. E., Gimnig, W., Hawley, T., & Lehmann. (2005). Larval development of the molecular forms of *Anopheles gambiae* (Diptera: Culicidae) in different habitats: a transplantation experiment. *Journal of Medical Entomology, 42,* 548-553.

Diatchenko, L., Lau, Y-F. C., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyano, V. S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., & Siebert, P. D. (1996). Suppression subtractive hybrizidation: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Science of the United States of America, 93,* 6025-6030.

Dienes, T., Pastor, S.J., Schurch, S., Scott, J. R., Yao, J., Cui, S., & Wilkins, C. L. (1996). Fourier Transform Mass Spectrometry-Advancing Years (1992-Mid 1996). *Mass Spectrometry Review, 15,* 163-211.

Dimopoulos, G., (2003). Insect immunity and its implication in mosquito-malaria interactions. *Cell Microbiology, 5,* 3-14.

Djouaka, R. F., Bakare, A. A., Bankole, H. S., Doannio, J. M., Coulibaly, O. N., Kossou, H., Tamo, M., Basene, H. I., Popoola, O. K., & Akogbeto, M. C. (2007). Does the spillage of petroleum products in *Anopheles* breeding sites have an impact on the pyrethroid resistance? *Malaria Journal, 6,* 159-160.

Djouaka, R. F., Bakare, A. A., & Coulibaly, O. N. (2008). Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant

populations of *Anopheles gambiae s. s*. from Southern Benin and Nigeria. *BMC Genomics, 9,* 538.

Doherty, M. K., Whitehead, C., McCormack, H., Gaskell, S. J., & Beynon, R. J. (2005). Proteome dynamics in complex organisms: Using stable isotopes to monitor individual protein turnover rates. *Proteomics, 5*(2), 522-533.

Dong, M. W. (1992). Tryptic mapping by revesed phase liquid chromatography. *Advances in Chromatography, 32,* 21-51.

Dong, M. W. (2006). *Modern HPLC for Practicing Scientists.* John Wilt & Sons, NJ, USA.

Donnelly, M. J., McCall, P. J., Lengeler, C., Bates, I., D'Alessandro, U., Barnish, G., Konradsen, F., Klinkenberg, E., Townson, H., Trape, J. F., Hastings, I. M., & Mutero, C. (2005). Malaria and urbanization in sub-Saharan Africa. *Malaria Journal*, *4*, 12-13.

Dorigatti, I., McCormack, C., Nedjati-Gilani, G., & Ferguson, N. M. (2017). Using *Wolbachia* for Dengue Control: Insights from modeling. *Trends in Parasitology, 34,* 102-113.

Dow, J. A., & Davies, S. A. (2006). The Malpighian tubule: rapid insights from post-genomic biology. *Journal of Insect Physiology, 52,* 365-378.

Duggan, D. J., Bittner, M., Chen, Y., Meltzer, P., & Trent, J. M. (1999). Expression profiling using cDNA microarrays. *Nature Genetics, 21,* 10-14.

Duguid, J. R. & Dinauer, M. C. (1990). Library subtraction of *in vitro* cDNA libraries to identify differentially expressed genes in scrapie infection. *Nucleic Acid Research, 18,* 2789-27-92.

Dutta, P., Khan, A. M., Khan, S. A., Boah, J., Sharma, C. K., & Mahanta, J. (2011). Malaria control in a forest fringe area of Assam, India; a pilot study. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 105,* 327-332.

Duval, L., Nerrienet, E., Rousset, D., Mba, S. A. S., Houze, S., Fourment, M., Le Bras, J., Robert, F., & Ariey, F. (2009). Chimpanzee malaria parasites related to *Plasmodium ovale* in Africa. *Nucleic Acids Research, 19,* 7097-7104.

El-Aneed, A., Cohen, A., & Banoub, J. (2009). Mass spectrometry, Review of the Basics: Electrospray, MALDI and commonly used Mass analyzers. *Applied Spectroscopy Reviews, 44,* 210-230.

Eliuk, S. & Makarov, A. (2015). Evolution of Orbitrap Mass Spectrometry Instrumentation. *Annual Review of analytical Chemistry, 8,* 61-80.

Elliott, M. H., Smith, D. S., Parker, C. E., & Borchers, C. (2009). Current trends in quantitative proteomics. *Journal of Mass Spectrometry, 44*(12), 1637-1660.

Emre, I., Kayis, T., Coskun, M., Dursun, O., & Cogun, H. Y. (2013). Changes in antioxidative enzyme activity, glycogen, lipid, protein and malondialdehyde content in cadmium-treated *Galleria mellonella* larvae. *Annals of the Entomological Society of America, 106*(3):371-377.

Engholm-Keller, K., Hansen, T. A., Palmisano, G., & Larsen, M. R. (2011). Multidimensional strategy for sensitive phosphoproteomics incorporating protein Prefractionation combined with SIMAC, HILIC, and TiO_2 Chromatography Applied to Proximal EGF Signaling. *Journal of Proteome Research, 10*(12), 5383-5397.

English, M., Waruiru, C., Amukoye, E., Murphy, S., Crawley, J., & Mwangi, I. (1996). Deep breathing in children with severe malaria: indicator of metabolic acidosis and poor outcome. *American Journal of Tropical Medicine and Hygiene, 55,* 521-524.

Essader, A. S., Cargile, B. J, Bundy, J. L., & Stephenson, J. L Jr. (2005). A comparison of immobilized pH gradient isoelectric focusing and strong-cation-exchange chromatography as a first dimension in shotgun proteomics. *Proteomics, 5*(1), 24-34.

Esvelt, K. M., Smiddler, A. L., Catteruccia, F., & Church, G. M. (2014). Concerning RNA-guided genedrives for the alteration of wild populations. *Elife 3,* e03401.

Everley, P. A., Krijgsveld, J., Zetter, B. R., & Gygi, S. P. (2004). Quantitative cancer proteomics: stable isotope labelling with amino acids in cell culture (SILAC) as a tool for prostate cancer research. *Molecular and Cellular Proteomics, 3,* 729-735.

Fegan, G.W., Noor, A. M., Akhwale, W. S., Cousens, S., & Snow, R. W. (2007). Effect of expanded insecticide-treated bed net coverage on child survival in rural Kenya: A longitudinal study. *The Lancet, 370,* 1035-1039.

Felix, R. C., Muller, P., Ribeiro, V., Ranson, H., & Silveira, H. (2010). *Plasmodium* infection alters *Anopheles gambiae* detoxification gene expression. *BMC Genomic*s *11,* 312.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science, 246*(4926), 64-71.

Ferguson, J. E. (1990). *The Heavy Elements*: Chemistry, Environmental Impact and Health Effects (Oxford: Pergamon Press).

Ferguson, N. M., Kien, D. T. H., Clapham, H., Aguas, R., Trung, V. T., Chau, T. N.B., Popovici, J., Ryan, P. A., O`Neil, S. L., McGraw, E. A., Long, V. T., Dui, L. T., Nguyen, H. L., Chau, N. V. V., Wills, B., & Simmons, C. P. (2015). Modeling the impact on virus transmission of *Wolbachia* mediated blocking of dengue virus. *Science Translational Medicine, 7* (279), 279ra37.

Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., & White, F. M. (2002). Phosphoproteome Analysis by Mass Spectrometry and its Application to *Saccharomyces Cerevisaiae*. *Nature Biotechnology, 20*(3), 301-305.

Fields, S. (2001). Proteomics. Proteomics in genome land. *Science, 291,* 1221-1224.

Fillinger, U., Knols, B., & Becker, N. (2003). Efficacy and efficiency of new *Bacillus thuringiensis var.israelensis* and *Bacillus sphaericus* formulations against Afrotropical Anophelines in Western Kenya. *Tropical Medicine and International Health, 8,* 37-47.

Flores, H. A., & O'Neill, S. L. (2018). Controlling vector-borne diseases by releasing modified mosquitoes. *Nature Reviews. Microbiology, 16*(8), 508-518.

Focks, D. A., Sackett, S. R., Dame, D. A., & Bailey, D. L. (1985). Effect of weekly releases of *Toxorhynchites amboinensis* (Doleschall) on *Aedes aegypti (L.)* (Diptera: Culicidaee) in New Orleans, Louisiana. *Journal of Economic Entomology, 78,* 622-626.

Ford, H. R., & Green, E. (1972). Laboratory rearing of *Anopheles albimanus. Mosquito News, 32*, 509-513.

Forstner, U. (1995). *Land contamination by metals: global scope and magnitude of the problem.* Pp 1-33. In: H. E. Allen, C. P. Huang, G. W. Bailey, and A. R. Bowers (eds). Metal speciation and contamination of soil. CRC, Boca Raton.

Foster, L., de Hoog C., Zhang, Y., Xie, X., Mootha, V., & Mann, M. (2006). A mammalian organelle map by protein correlation profiling. *Cell, 125* (1), 187 -199.

Gakuya, D. W., Itonga, S. M., Mbaria, M., Muthee, J. K., & Musau, J. K. (2013). Ethnobotanical survey of biopesticides and other medicinal plants traditionally used in Meru Central District of Kenya. *Journal of Ethnopharmacology, 145*(2), 547-553.

Gan, C. S., Guo, T., Zhang, H., Lim, S. K., & Size, S. K. (2008). A comparative study of Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) versus SCX-

IMAC-Based Methods for phosphopeptide Isolation=Enrichment. *Journal of proteome Research, 7*(11), 4869-4877.

Gantz, V. M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V. M., Bier, E., & James, A. A. (2015). Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proceedings of the National Academy of Science of the United States of America, 112,* E6736-E6743.

Gao, J., Opiteck, G. J., Friedrichs, M. S. Donge, A. R., & Hefta S. R. (2003). Changes in the Protein Expression of Yeast as a function of Carbon Source. *Journal of Proteome Research, 2,* 643-649.

Gelderman, M. P., & Simak, J. (2008). Functional proteomics. *Methods, 484,* 79-93.

Georghiou, G. P., & Wirth, M. C. (1997). Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *Israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). *Applied Environmental Microbiology, 63,* 1095-1101.

Gerberg, E. J., Barnard, D. R., & Ward, R. A. (1994). *Manual for mosquito rearing and experimental techniques,* revised edition. American Mosquito Control Association, Inc., Lake Charles

Gewartowski, K., Cuellar, J., Dziembowski, A., & Valpuesta, J. M. (2012). The yeast THO complex forms a 5-subunit assembly that directly interacts with active chromatin. *BioArchives, 2*(4), 134-137.

Gilar, M., Olivova, P., Daly, A. E., & Gebler, J. C. (2005a). Orthogonality of separation in two dimensional liquid chromatography. *Analytical Chemistry, 77*(19), 6426-6434.

Gilar, M., Olivova, P., Daly, A. E., & Gebler, J. C. (2005b). Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in the first and second separation dimensions. *Journal of Separation Science, 28*(14), 1694-1703.

Gillies, M. T. (1955). The pre-gravid phase of ovarian development in *Anopheles funestus. American Journal of Tropical Medicine and Parasitology, 49,* 320-325.

Gillies, M. T., & de Meillon, B. (1968) The Anophelinae of Africa South of the Sahara (Ethiopian zoogeographical region) Johannesburg: The South African Institute for Medical Research, 54.

Gillies, M. T., & Coetzee, M. (1987). A supplement to the Anophelinae of Africa South of the Sahara Johnnesburg: *The South African Institute for Medical Research*, 54.

Gimnig, J. E., Ombok, M., Kamau, L., & Hawley, W. (2001). Characteristics of larva *anopheline* (Diptera: Culicidae) habitats in Western Kenya. *Journal of Medical Entomology, 38,* 282-288.

Giraldo-Calderon, G. I., Emrich, S. J., MacCallum, R. M., Maslen, G., Dialynas, E., Topalis, P., Ho, N., Gesing, S., VectorBase, C., & Madey, G. (2015). VectorBase: an updated bioinformatics resource for invertebrate vectors and other organisms related with human diseases. *Nucleic Acids Research, 43,* D707-D713.

Gisbert, C. R., Ros, A., de Haro, D. J., Walker, M. P., Bemal, R., Serrano, P., & Navarro-Avino, J. (2003). A plant genetically modified that accumulates Pb is especially promising for phytoremediation. *Biochemistry and Biophysics Research Communication, 303,* 440-445.

Golawska, S., Leszczynski, B., & Oleszek, W. (2006). Effect of low and high-saponin lines of alfalfa on pea aphid. *Journal of Insect Physiology, 52,* 737-743.

Gonza´lez-Ortega, O., Porath, J., & Guzma´n, R. (2012). Adsorption of peptides and Small proteins with Control Access polymer permeation to Affinity Binding Sites. Part 1: Polymer permeation-Immobilized Metal Ion Affinity Chromatography Separation Adsorbents with Polyethylene Glycol and Immobilized Metal Ions. *Journal of Chromatography A, 1227,* 115-125.

González, J. A., García-Barriuso, M., Gordaliza, M., & Amich, F. (2011). Traditional plant-based remedies to control insect vectors of disesase in the Arribes Del Duero (Western Spain): An ethnobotanical study. *Journal of Ethnopharmacology,* 138(2), 595-601.

Gorg, A., Postel, W., & Gunther, S. (1988). The current state of two dimensional electrophoresis with immobised pH gradients. *Electrophoresis, 9*(9), 531-546.

Gorg, A., Postel, W., Friedrich, C., Kuick, R., Strahler, J., & Hanash, S. (1991). Temperature-dependent spot positional variability in two dimensional polypeptide patterns. *Electrophoresis, 12*(9), 653-658.

Gorg, A., Weiss, W., & Dumn, M. (2004). Current two-dimensional electrophoresis technology for proteomics. *Proteomics, 4*(12), 3665-3685.

Gorg, A., Drews, O., Luck, C., Weiland, F., & Weiss, W. (2009). 2-DE with IPGs. *Electrophoresis, 30*(1), S122-S132.

Gou, Y., Li, Z., Fan, R., Qiu, Z., Wang, L., Wang, C., & Wang, Y. (2020). Ethnobotanical survey of plants traditionally used against hematophagous invertebrates by ethnic groups in the mountainous area of Xishuangbanna, Southwest China. *Plant Diversity*, https://doi.org/10.1016/j.pdl.2020.07.009.

Gouw, J.W., Krijgsveld, J., & Heck, A. J. (2010). Quantitative proteomics by metabolic labeling of model organisms. *Molecular and Cellular Proteomics, 9*(1), 11-24.

Granja, A. C., Machungo, F., Gomes, A., Bergstrom, S., & Brabin, B. (1998). Malaria-related maternal mortality in urban Mozambique. *Annals of Tropical Medicine and Parasitology, 92*, 257-263.

Graves, P. & Haystead, T. (2002). Molecular biologist's guide to proteomics. *Microbiology and Molecular Biology Review, 66*(10), 39-63.

Greenwood, B. M., Fidock, D. A., Kyle, D. E., Kappe, S. H. I., Alonso, P. L., Collins, F. H., & Duffy, P. E. (2008). Malaria: progress, perils and prospects for eradication. *Journal of Clinical Investigation, 118,* 1266-1276.

Griffin, L. F., & Knight, J. M. (2012). A review of the role of fish as biological control agents of disease vector mosquitoes in mangrove forests: Reducing human health risks while reducing environmental risk. *Wetland Ecological Management, 20,* 243-252.

Guo, Z. J., Kang, S., Zhu, X., Xia, J. X., Wu, Q. J., & Wang, S. L. (2015). The novel ABC transporter ABCH l is a potential target for RNAi-based insect pest control and resistance management. *Scientific Report, 5,* 13728.

Gupta, S., Pfannkoch, E., & Regnier, F. E. (1983). High-performance cation-exchange Chromatography of proteins. *Analytical Biochemistry, 128,* 196-201.

Hales, S., & van Panhuis, W. (2005). A new strategy for dengue control. *The Lancet, 365,* 551-552.

Halim, M., Conte, P., & Piccolo, A. (2003). Potential availability of heavy metals to phytoextraction from contaminated soils induced by exogenous humic substances. *Chemosphere, 52,* 265-275.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C., Karsanos, D., Gribble, M., Baker, D., Marois, E., Russell, S., Burt, A., Windbichler, N., Crisanti, A., & Nolan, T. (2016). A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *National Biotechnology, 34,* 78-83.

Han, Y., Gao, S., Muegge, K., Zhang, W., & Zhou, B. (2015). Advanced Applications of RNA sequencing and challenges. *Bioinformatics and Biology Insights, 9,* 29-46.

Hara, E., Kato. T., Nakada, S., Sekiya, S., & Oda, K. (1991). Subtractive cDNA cloning using oligo(dT) 30-latex and PCR: Isolation of cDNA clones specific to undifferentiated human embryonal carcinoma cells. *Nucleic Acid Research, 19,* 7097-7104.

Hare, L. (1992). Aquatic insects and trace metals: Bio-availability, bio-accumulation and toxicity. *Critical Review of Toxicology*, *22,* 327 -369.

Hay, S. I., Rogers, D. J., Toomer, J. F., & Snow, R.W. (2000). Annual *Plasmodium falciparum* entomological inoculation rates (EIR) across Africa: literature survey, Internet access and review. *Transactions of Royal Society Tropical Medicine Hygiene*, *94,* 113-127.

Hay, I. S., Guerra, A. C., Tatem, J. A., Noor, M. A., & Snow, W. R. (2004). The global distribution and population at risk of malaria past present and future. *Lancet Infectious Diseases, 4,* 327-336.

Hay, S. I., Guerra, C. A., Tatem, A. J., Atkinson, R. M., & Snow, R.W. (2005). Urbanization, malaria transmission and disease burden in Africa. *National Review of Microbiology*, *3,* 81-90.

He, F., Hendrickson, C. L., & Marshall, A. G. (2001). Baseline mass resolution of peptide isobars: A record for molecular mass resolution. *Analytical Chemistry, 73,* 647-650.

Hedrick, S. M., Cohen, D. I., Neilson, E. A., & Davis, M. M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature, 308,* 149-153.

Heller, M., Mattou, H., Menzel, C., & Yao, X. (2003). Trypsin catalyzed ^{16}O-^{18}O Exchange for comparative proteomics: tandem mass spectrometry comparison using MALDI-TOF, ESI-QTOF and ESI-ion trap mass spectrometers. *Journal of American Society of Mass Spectrometry, 14,* 704-718.

Henzel, W. J., Billeci, T. M., Stults, J. T., Wong, S. C., Grimley, C., & Watanabe, C. (1993). Identifying proteins from two dimensional gels by molecular mass searching of peptide fragments in proteins sequence databases. *Proceedings of the National Academy of Sciences of the United States of America*, *90,* 5011-5015.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., & Werren, J. H. (2008). How many species are infected with *Wolbachia*? A statistical analysis of current data. *Federation of European Microbiological Societies, Microbiology Letters, 281,* 215-220.

Hochstrasser, M. (1997). Ubiquitin-dependent protein degradation. *Annual Review of Genetics, 30,* 405-439.

Hollenstein, K., Frei, D. C., & Locher, K. P. (2007). Structure of an ABC transporter in complex with its binding protein. *Nature, 446,* 213-216.

Hong, S. M., Yamashita, J., Mitsunobu, H., Uchino, K., Kobayashi, I., & Sezutsu, H. (2010). Efficient soluble protein production on transgenic silkworms expressing cytoplasmic chaperones. *Applied Microbiology and Biotechnology, 87,* 2147-2156.

Howbrook, D. N., van der Valk, A. M., O'Shaughnessy, M. C., Sarker, D. K., Baker, S. C. & Lyoyd, A. W. (2003). Developments in microarray technologies. *Drug Discovery Today, 8,* 642-651.

Hviid, L., & Salanti, A. (2007). VAR2CSA and protective immunity against pregnancy associated *Plasmodium falciparum* malaria. *Parasitology, 134,* 1871-1876.

Hwang, I-T., Kim, Y. J., Kim, S-H., Kwak, C-I., Gu, Y-Y., & Chun, J-Y. (2003). Annealing control primer system for improving specificity of PCR amplification. *BioTechniques, 35,* 1180-1184.

Hwang, K. C., Cui, X. S., Park, S. P., Shin, M. R., Park, S. Y., Kim, E. Y., & Kim, N. H. (2004). Identification of differentially regulated genes in bovine blastocysts using an annealing control primer system. *Molecular Reproduction and Development, 69,* 43-51.

Hwang, K. C., Lee, H. Y., Cui, S. S., Kim, J. H., & Kim, N. H. (2005). Identification of Maternal mRNAs in porcine parthenotes at the 2-cell stage: A comparison with the blastocyst stage. *Molecular Reproduction and Development, 70,* 314-323.

Janciauskiene, S. (2001). Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles. *Acta Tropica, 1535,* 221-235.

Jiang, C., Wechuck, J. B., Goins, W. F., Krisky, D. M., Wolfe, D., Ataai, M. M., & Glorioso, J. C. (2004). Immobilizd cobalt affinity chromatography provides a novel, efficient method for herpes simplex virus type 1 gene vector purification. *Journal of Virology, 78,* 8994-9006.

Jiang, C., Glorioso, J. C., & Ataai, M. (2006). Presence of imidazole in loading buffer prevents formation of free radical in immobilized metal affinity chromatography and dramatically improves the recovery of herpes simplex virus type 1 therapy vectors. *Journal of Chromatography A, 1121,* 40-45.

Johnson, K. L., & Muddiman, D. C. (2004). A method for calculating $^{16}O/^{18}O$ peptide ion ratios for the relative quantification of proteomes. *Journal of the American Society for Mass Spectrometry, 15*(4), 437-445.

Jones, C. M., Toe, H. K., Sanou, A., Namountougou, M., Hughes, A., & Diabate, A. (2012). Additional selection for insecticide resistance in urban malaria vectors: DDT resistance in *Anopheles arabiensis* from BoboDioulasso, Burkina Faso. *PLoS One, 7*(9), e45995.

Jung, J., Yoon, T., Choi, E., & Lee, K. (2002). Interaction of cofilin with triosephosphate isomerase contributes glycolytic fuel for Na, K-ATPase via Rho-mediated signaling pathway. *Journal of Biological Chemistry, 277,* 48931-48937.

Kabiru, E. W., Mbogo, C. M., Muiruri, S. K., Ouma, J. H., Githure, J. I., & Beier, J. C. (1997). Sporozoite loads of naturally infected *Anopheles* in Kilifi District, Kenya. *Journal of American Mosquito Control Association, 13,* 259-262.

Kafel, A., Bednarska, K., Augustyniak, M., Witas, I., & Szulinska, E. (2003). Activity of glutathione S-transferase in *Spodoptera exigua* larvae exposed to cadmium and zinc in two subsequent generations. *Environment International, 28,* 683-686.

Kalra, H., Adda, C.G., Liem, M., Ang, C. S., Mechler, A., Simpson, R. J., Hulett, M. D., & Mathivanan, S. (2013). Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. *Proteomics, 13*(22), 3354-3364.

Kamareddine, L. (2012). The biological control of the malaria vector. *Toxins, 4,* 748-767.

Kamau, N. J. (2002). Heavy metal distribution and enrichment at Port-Reitz Creek, Mombasa, Western Indian Ocean. *Journal of Marine Science, 1,* 65-70.

Kanost, M. R., Kawooya, J. K, Ryan, R. D., van Heusden, M. C., & Ziegler, R. (1990). Insect hemolymph proteins. *Advances in Insect Physiology, 22,* 299-366.

Karakach, T. K., Flight, R. M., Douglas, S. E., & Wentzell, P. D. (2010). An introduction to DNA microarrays for gene expression analysis. *Chemometrics and Intelligent Laboratory Systems, 104,* 28-52.

Karas, M., & Hillenkamp, F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10000 Daltons. *Analytical Chemistry, 60*(20), 2299-2301.

Karungu, S., Atoni, E., Ogalo, J., Mwaliko, C., Agwanda, B., Yuan, Z., & Hu, X. (2019). Mosquitoes of Etiological Concern in Kenya and Possible Control Strategies. *Insects, 10*(6), 173

Kats, L. B., & Ferrer, R. P. (2003). Alien predators and amphibian declines: Review of two decades of science and the transition to conversation. *Diversity and Distributions, 9,* 99-110.

Kaur, G., Ali, S. A., Kumar, S., Mohanty, A. K., & Behare, P. (2017). Label-free quantitative proteomic analysis of *Lactobacillus fermentum* NCDC 400 during bile salt exposure. *Journal of Proteomics, 167,* 36-45.

Kay, B. H., Tuyet, H. T., Le, N. H., Quy, T. M., Nam, V. S., Hang, P.V., Yen, N. T., Hill, P.S., Vos, T., & Ryan, P. A. (2010). Sustainability and cost of a community-based strategy against *Aedes aegypti* in Nothern and Central Vietnam. *American Journal of Tropical Medicine and Hygiene, 82,* 822-830.

Keiji, K. & Takashi, I. (2008). Mass spectrometry-based approaches toward absolute quantitative proteomics. *Current Genomics, 9*(4), 263-274.

Kenig, M., Peternel, S., Gaberc-Porekar, V., & Menart, V. (2006). Influence of the protein oligometricty of final yield after affinity tag removal in purification of recombinant proteins. *Journal of Chromatography A, 1101,* 293-306.

Kettle, D. S. (1992). *Medical and Veterinary Entomology.* Centre for Agriculture and Biosciences International. Wallingford, UK, 99-136.

Khanam, M. L. A., Talukder, D., & Hye, M. A. (2006). Toxic and repellent action of sugarcane bagasse-based lignin against some stored grain insect pests. *University Journal of Zoology* Rajshahi University, *25,* 27-30.

Khelurkar, V. C., Ingle, K. P., & Padole, D. A. (2017). DNA Microarray: Basic Principle and its Applications. *Trends in Biosciences, 10*(2), 488-490.

Kidane, D., Tomass, Z., & Dejene, T. (2013). Community knowledge of traditional mosquito repellent plants in Kola Temben district, Tigray, northern Ethopia. *Scientific Research and Essays, 8*(24), 1139-1144.

Killeen, G. F., Fillinger, U., & Knols, B. G. J. (2002). Advantages of larval control for African malaria vectors: Low mobility and behavioral responsiveness of immature mosquito stages allow high effective coverage. *Malaria Journal, 1,* 8-9.

Killeen, G. F., Seyoum, A., & Knols, B. G. J. (2004). Rationalizing historical successes of Malaria control in African terms of Mosquito resource availability and management. *American Journal of Tropical Medicine and Hygiene, 7,* 87-93.

Kim, K. A., Chakraborti, T., Goldstein, G. W., & Bressler, J. P. (2000). Immediate early gene expression in PC12 cells exposed to lead: Requirement for protein kinase C. *Journal of Neurochemistry, 74,* 1140-1146.

Kim, Y. J., Kwak, C. I., Gu, Y. Y., Hwang, I. T., & Chun, J. Y. (2004a). Annealing control primer system for identification of differentially expressed genes on agarose gels. *Biotechniques, 36,* 424-430.

Kim, H., Page, G., & Barnes, S. (2004b). Proteomics and mass spectrometry in nutrition research. *Nutrition, 20*(1), 155-165.

Kim, S., Mischerikow, N., & Bandeira, N. (2010). The generating function of CID, ETD and C1D/ETD pairs of tandem mass spectra: applications to database search. *Molecular and Cellular Proteomics, 9*(12), 2840-2852.

Kimani, N. G. (2007). *Implications of the Dandora municipal dumping site in Nairobi, Kenya.* Environmental Pollution and Impacts on Public Health. Kenya: United nations Environment programme.

Kingdon, K. H. (1923). A method for the neutralization of electron space charge by positive ionization at very low gas pressures. *Physics Review, 21,* 408-418.

Kinuthia, G. K., Ngure, V., Beti, D., Lugalia, R., Wangila, A., & Kamau, L. (2020). Levels of heavy metals in waste water and soil samples from open drainage channels in Nairobi, Kenya: Community health implication. *Scientific Reports Nature, 10,* 8434.

Kistler, K. E., Voosshall, L. B., & Matthews, B. J. (2015). Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti, Cell Reports, 11,* 51-60.

Kitron, U., & Spielman, A. (1989). Suppression of transmission of malaria through source reduction: anti-anopheline measures applied in Israel, the United States and Italy. *Review of Infectious Diseases, 11,* 391-406.

Klassen, W., & Curtis, C. F. (2005). History of the sterile insect technique. In Dyck, V. A., Hendrichs, J., Robinson, A.S., eds. Principles and practice in Area-wide integrated pest management. *Springer, 801,* 3-36.

Klerks, P. L., & Weis, J. S. (1987). Genetic adaptation to heavy metals in aquatic organisms: A review. *Environmental Pollution, 45,* 173-205.

Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. *Human genetics, 26*(3), 231-243.

KNBS. (2017). *Nairobi: Kenya Urbanization Review. (2016*). Republic of Kenya Report No: AUS8099.

Knols, B. G. J., Hood-Nowotny, R. C., Bossin, H., Franz, G., Robinson, A., Mukabana, W. R., & Kemboi, S. K. (2006). GM sterile mosquitoes: a cautionary note. *Nature Biotechnology, 24*(9), 1067-1068.

Köhler, A., & Hurt, E. (2007). Exporting RNA from the nucleus to the cytoplasm. *Nature Review of Molecular and Cell Biology, 8,* 761-773.

Kolch, W., Mischak, H., & Pitt, A. R. (2005). The molecular make-up of a tumor: proteomics in cancer research. *Clinical Science, 108*(5), 369-383.

Konan, Y.L., Sylla, M.S., Doannio, J. M. C., & Traoré S. (2003). Comparison of the effect of two excipients (Karite Nut butter and Vaseline) on the efficacy of *Cocos nucifera, Elaeis guineensis* and *Carapa procera* oil-based repellents formulations against mosquitos biting in Ivory Coast. *Parasites, 10*(2), 181-184.

Koomen, J., Hawke, D., & Kobayashi, R. (2005). Developing an understanding and introduction to biology of proteomics: Mass spectrometry. *Cancer Investigation, 23*(1), 47-59.

Kopaciewicz, W. & Regnier F. E. (1983). Mobile phase selection for the high-perfomance ion-exchange chromatography of proteins. *Analytical Biochemistry, 133,* 251.

Korsloot, A., Van Gestel, C. A. M., & van Straalen, N. M. (2004). *Environmental Stress and Cellular Response in Arthropods.* CRC Press, Boca Raton.

Krantzberg, G. I., & Stokes, P. M. (1990). Metal concentration and tissue distribution in larvae of *Chironomus* with reference to X-ray microprobe analysis. *Environmental Contamination and Toxicology, 19,* 84-93.

Kruger, M., Moser, M., Ussar, S., Thievessen, I., Luber, C. A., Forner, F., Schmidt, S., Zanivan, S., Fassler, R., & Mann, M. (2008). SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell, 134*(2), 353-364.

Krusemark, C. J., Frey, B. L., Belshaw, P. J. & Smith, L. M. (2009). Modifying the charge state distribution of proteins in Electrospray ionization Mass Spectrometry by Chemical Derivatization. *Journal of American Society of Mass Spectrometry, 20,* 1617-1625.

Kukurba, K. R., & Montgomery, S. B. (2015). RNA sequencing and Analysis. *Cold Spring Harbor Protocols, 11,* 951-969.

Kumar, R., & Hwang, J. S. (2006). Larvicidal efficiency of aquatic predators: A perspective for mosquito biocontrol. *Zoology Studies, 45,* 447-466.

Kunkel, L. M., Manaco, A. P., Middlesworth, W., Ochs, H. D., & Latt, S. A. (1985). Specific cloning of DNA fragments absent from the DNA of a male patient with an X chromosome deletion. *Proceedings of the National Academy of Science of the United States of America, 82,* 4778-4782.

Kweka, E. J., Mosha, F., Lowasa, A., Mahande, A. M., Kitau, J., Matowo, J., Mahande, M. J., Massenga, C. P., Tenu, F., Feston, E., Lyatuu, E. E., Mboya, M. A., Mndeme, R., Chuwa, G., & Temu, E. A. (2008). Ethnobotanical study of some of mosquito repellent plants in north-eastern Tanzania. *Malaria Journal, 7*:152.

Kweka, E. J., Zhou, G., Gilbreath, T. M., Afrane, Y., Nyindo, M., Githeko, A.K., & Yan, G. (2011). Predation efficiency of *Anopheles gambiae* larvae by aquatic predators in Western Kenya Highlands. *Parasites and Vectors, 4,* 128.

Lacey, L. A., & Undeen, A. H. (1986). Microbial control of black flies and mosquitoes. *Annual Review of Entomology, 31,* 265-296.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680-685.

Lajis, N. H., Hussein, M. Y., & Toia, R. F. (1985). Extraction and Identification of the main compound present in *Elaies guineensis* flower volatiles. *Pertanika, 8*(1), 105-108.

Lam, P. H., Boon, C. S., Ying, N. Y., & Benjamin, S. (2010). *Aedes albopictus* control with spray application of *Bacillus thuringiensis israelensis,* strain AM 65-52. Southeast Asian *Journal of Tropical Medicine and Public Health, 41,* 1071-1081.

Lamar, E. E., & Palmer, E. (1984). Y-encoded, species-specific DNA in mice: evidence that the Y chromosome exist in two polymorphic forms in inbred strains. *Cell, 37,* 171-177.

Lanzaro, G. C., Toure, Y. T., Carnahan, J., Zheng, L., Dolo, G., Traore, S., Petrarca, V., Vernick, K. D., & Taylor, C. E. (1998). Complexities in the genetic structure of *Anopheles*

gambiae populations in West Africa as revealed by microsatellite DNA analysis. *Proceedings of the National Academy of Science of the United States of America, 95,* 14260-14265.

Lasaosa, M., Delmote, N., Huber, C. G., Melchior, K., Heizle, E., & Tholey, A. (2009). A 2D reversed-phase × ion pair reversed-phase HPLC-MALDI TOF/TOF-MS approach for shotgun proteome analysis. *Analytical and Bionalytical Chemistry, 393*(4), 1245-1256.

Lashkari, D. A., DeRisi, J. L., McCusker, J. H., Namath, A. F., Gentile, C., Hwang, S. Y., Brown, P. O., & Davis, R. W. (1997). Yeast microarrays for genome wide parallel genetic and gene expression analysis. *Proceedings of the National Academy of Science of the United States of America, 94,* 13057-13062.

Lecchi, P., & Abramson, F P. (1998). Analysis of biopolymers by size-exclusison chromatography-mass spectrometry. *Journal of Chromatography A, 828*(1-2), 509-513.

Lecchi, P., & Abramson, F. P. (1999). Size exclusion chromatography-chemical reaction interface mass spectrometry: a perfect match". *Analytical Chemistry, 71*(14), 2591-2955.

Lengeler, C. (2004). Insecticide-treated bed nets and curtains for preventing malaria. *The Cochrane Database of Systematic Reviews, 2,* CD000363.

Leung, Y. F., & Cavalieri, D. (2003). Fundamentals of cDNA microarray data analysis. *Trends in Genetics, 19,* 649-659.

Li, Y. J., Oliveira, S. A., Xu, P., Martin, E. R., Stenger, J. E., Scherzer, C. R., Hauser, M. A., Scott, W. K., Small, G. W., & Nance, M. A. (2003). Glutathione S-transferase omega-l modifies age-at-onset of Alzheimer disease and Parkinson disease. *Human Molecular Genetics, 12,* 3259-3267.

Liang, M., Briggs, A. G., Rute, E., Greene, A. S., & Cowley, A. W. Jr. (2003). Quantitative assessment of the importance of dye switching and biological replication in cDNA microarray studies. *Physiological Genomics, 14,* 199-207.

Liao, V. H., & Freedman, J. H. (1998). Cadmium-regulated genes from the nematode *Caenorhabditis elegans.* Identification and cloning of new cadmium-responsive genes by differential display. *Journal of Biological Chemistry, 273,* 31962-31970.

Lijun, L., Xuemeia, L., Yaping, G., & Enbo, M. (2005). Activity of the enzymes of the antioxidative system in cadmium treated *Oxya chinensis* (Orthoptera Acridoidae) *Environmental Toxicology and Pharmacology, 20,* 412-416.

Lin, S-L., Chang, D., Miller, J. D., & Ying, S-Y. (2007). Subtractive Hybridization with Covalently Modified Oligonucletides. *Nucleic Acids Hybridization Modern Applications*, *6,* 167-186.

Lindsay, S., Ewald, J., Samung, Y., Apiwathnasorn, C., & Nosten, F. (1998). Thanaka (*Limonia acidissima*) and DEET (Di-methyl benzamide) mixture as a mosquito repellent for use by Karen women. *Medical and Veterinary Entomology, 12,* 295-301.

Littman, D. R., Thomas, Y., Maddon, P. J., Chess, L., & Axel R. (1985). The isolation and sequence of the gene encoding T8: A molecule defining functional classes of T lymphocytes. *Cell, 40,* 237-246.

Liu, H., Lin, D., & Yates, J. R. (2002). Multidimensional separations for protein/peptide analysis in the post-genomic era. *Biotechniques, 32*(4), 898-902

Lo, W. L., Mok, K. L., Yu, S. D., Ming, Y. (2018). Which insect repellents should we choose? Implications from results of local market survey and review of current guidelines. *Hong Kong Journal of Emergency Medicine, 25*(5), 272-280.

Lopez-Martinez, G., Benoit, J. B., Rinehart, J. P., Elnitsky, M. A., Lee, R. E., & Denlinger, D. L. (2009). Dehydration, rehydration and overhydration alter patterns of gene expression in the Antarctic midge, *Belgica antarctica*. *Journal of Comparative Physiology,* 179, 481-491.

Louca, V., Lucas, M. C., Green, C., Majambere, S., Fillinger, U., & Lindsay, S. W. (2009). Role of fish as predators of mosquito larvae on the floodplain of the Gambia river. *Journal of Medical of Entomology, 46,* 546-556.

Luo, M., Cao, H., Mei., Fan, Y. Y., Zhou, X. C., Chen, J. X., Chung, H., & Wei, H. Y. (2019). Bioaccumulation of cadmium affects development, mating behaviour and fecundity in the Asia Corn Borer, *Ostrinia furnacalis. Insects, 11,* 7.

Lyupina, Y. V., Dmitrieva, S. B., Timokhova, A. V., Beljelarskaya, S. N., Zatsepina, O. G., & Evgen'ev, M. B. (2010). An important role of the heat shock response in infected cells for replication of baculo viruses. *Journal of Virology, 406,* 336-341.

Machtejavas, E., John, H., Wagner, K., Standkler, L., Marko, V. G., Forssmann, W. F., Bischoff R., & Unger, K. K. (2004). Automated multi-dimensional liquid chromatography: sample preparation and identification of peptides from human blood filtrate. *Journal of Chromatography B, 803,* 121-130.

Maddon, P. J., Littman, D. R., Godfrey, M., Maddon, D. E., Chess, L., & Axel, R. (1985). The isolation and nucleotide sequence of a cDDA encoding the T cell suface protein T4: A new member of the immunoglobin gene family. *Cell, 42,* 93-104.

Magesa, S. M., Lengeler, C., de Savigny, D., Miller, J. E., Njau, R. J., Kramer, K., Kitua, A., & Mwita, A. (2005). Creating an enabling environment for taking insecticide-treated nets to national scale: the Tanzanian experience. *Malaria Journal***,** *4,* 34-35.

Maia, M. F., & Moore, S. J. (2011). Plant-based insect repellents: A review of their efficacy, development and testing. *Malaria Journal, 10,* S11.

Makarov, A. (2000). Electrostatic axially harmonic orbital trapping: A high-perfomance technique of mass analysis. *Analytical Chemistry, 72,* 1156-1162.

Manadas, B., English, J. A., Wynne, K. J., Cotter, D. R., & Dunn, M. J. (2009). Comparative analysis of OFFGel, strong cation exchange with pH gradient, and RP at high for first-dimensional separation of peptides from a membrane-enriched protein fraction. *Journal of Proteomics, 9*(22), 5194-5198.

March, R. E. (1997). An introduction to quadrupole ion trap mass spectrometry. *Journal of Mass Spectrometry, 32*(4), 351-369.

Marguerat, S., Wilhelm, T., & Bahler, J. (2008). ®Next-generation sequencing: Applications beyond genomes. *Biochemical Society Transactions, 36,* 1091-1096.

Marshall, A. G. & Guan, S. (1996). Advantages of High Magnetic field for FT-ICR Mass Spectrometry. *Mass Spectrometry, 10,* 1819-1823.

Marshall, A. G., Hendrickson, C. L., & Jackson, G. S. (1998). Fourier Transform Ion Cyclotron Resonance Mass Spectrometry: *A primer Mass Spectrometry Review, 17,* 1-35.

Marten, G. G., Astaiza, R., Suarez, M. F., Monje, C. & Reid, J. W. (1989). Natural control of Larval *Anopheles albimanus* (Diptera: Culicidae) by the predator *mesocyclops* (Copepoda: Cyclopoida). *Journal of Medical Entomology, 26,* 624-627.

Martin, M. H., & Coughtrey P. J. (1982). *Biological Monitoring of heavy metal pollution, 'land and air'* 475pp. London and New York.

Masiga, D. K., Turner, M., & Tait, A. (2000). Amplified restriction fragment length polymorphism in parasite genetics. *Parasitology Today, 16,* 350-353.

Matthys, B., N`Goran, E. K., Kone, M., Koudou, B. G., Vounatsou, P., Cisse, G., Tschannen, A. B., Tanner, M., & Utzinger, J. (2006). Urban agricultural land use and characterization of

mosquito larval habitats in a medium-sized town of Cote d'Ivoire. *Journal of Vector Ecology, 31,* 319-333.

Mattingly, K. S., Beaty, B. J., Mackie, R. S., McGaw, M., Carlson, J. D., & Rayms-Keller, A. (2001). Molecular cloning and characterization of a metal responsive *Chironomus tentans* alpha-tubulin cDNA. *Aquatic Toxicology, 54,* 249-260.

Mbogo, C. N. M., Snow, R. W., Khamala, C. P. M., Kabiru, E. W., Ouma, J. H., Githure, J. I., Marsh, K., & Beier, J. C. (1995). Relationships between *Plasmodium falciparum* transmission by vector populations and the incidence of severe disease at nine sites on the Kenyan coast. *American Journal of Tropical Hygiene, 52,* 201-206.

Mbogo, C. M., Mwangangi, J. M., Nzovu, J., Gu, W., Yan, G., Gunter, J .T., Swalm, C., Keating, J., Regens, J .L., Shililu, J. I., Githure, J. I., & Beier, J. C. (2003). "Spatial and temporal heterogeneity of *Anopheles* mosquitoes and *Plasmodium falciparum* transmission along the Kenyan coast". *American Journal of Tropical Medicine and Hygiene, 68*(6), 734-742.

McCall, M. N., Mukarami, P. N., Lukk, M., Huber, W., & Irizarry, R. A. (2011). Assesing affymetrix geneChip microarray quality. *BMC Bioinformatics, 12,* 137.

McCalley, D. V. (2005). Comparison of an organic polymeric colomn and a silica-based reversed-phase for the analysis of basic peptides by high-perfomance liquid chromatography. *Journal of Chromatography A, 1073*(1-2), 137-145.

McCalley, D. V. (2010). The challenges of the analysis of basic compounds by high performance liquid chromatography: some possible approaches for improved separations. *Journal of Chromatography A, 1217*(6), 858-880.

McGregor, I. A. (1984). Epidemiology, malaria and pregnancy. *American Journal of Tropical Medicine and Hygiene, 33,* 517-525.

McLafferty, F. W., & Bockhoif, F. M. (1978). Separation/identitication system for complex mixtures using mass separation and mass spectral characterization. *Analytical Chemistry, 50*(1), 69-76.

McLafferty, F. W. (1981). Tandem mass spectrometry. *Science, 214*(4518), 280-287.

McLafferty, F. W. (1994). High-Resolution Tandem FT Mass Spectrometry above 10 kDa. *Accounts of Chemical Research, 27,* 379-386.

McMaster, M. (2007). *HPLC: A Practical User's guide.* J. Wiley & Sons NJ, USA. *Journal of American Chemical Society, 129,* 3028-3029.

Megger, D. A., Bracht, T., Meyer, H. E., & Sitek, B. (2013). Label-free quantification in clinical proteomics. *Biochimica et Biophysica Acta, 1834*(8), 1581-1590.

Megy, K., Emrich, S. J., Lawson, D., Campbell, D., Dialynas, E., Hughes, D. S., Koscielny, G., Louis, C., Maccallum, R. M., & Redmond, S. N. (2012). VectorBase: improvements to a bioinformatics resource for invertebrate vector genomics. *Nucleic Acids Research, 40,* 729-734.

Menendez, C., Fleming, A. F., & Alonso, P. L. (2000). Malaria related anemia. *Parasitology Today, 16,* 469-476.

Metcalf, C. L., Flint, W. P., & Metcalf, R. L. (1962). *Destructive and useful insects: their habits and control. 4th Edition.* McGraw Hill Book Company, New York, 998-1009.

Meudt, H. M., & Clarke, A. C. (2007)."Almost forgotten or latest practice? AFLP applications, analyses and advances". *Trends in Plant Science, 12,* 106-117.

Michelmore, R. W., Paran, I., & Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Science of the United States of America, 88,* 9828-9832.

Minakawa, N., Mutero, C. M., Githure, J. I., Beier, J. C., & Yan, G. (1999). Spatial distribution and habitat characterisation of anopheline mosquito larvae in western Kenya. *American Journal of Tropical Medicine and Hygiene, 61,* 1010-1016.

Mireji, P. O., Keating, J., Kenya, E., Mbogo, C. M., Nyambaka, H., Osir, E., Githure, J., & Beier, J. (2006). Differential induction of proteins in *Anopheles gambiae sensu stricto* (Diptera: Culicidae) larvae in response to heavy metal selection. *International Journal of Tropical Insect Science, 26,* 214-226.

Mireji, P. O., Keating, J., Hassanali, A., Mbogo, C. M., Nyambaka, H., Kahindi, S., & Beier, J (2008). Heavy metals in mosquito larval habitats in urban Kisumu and Malindi, Kenya, and their impact. *Ecotoxicological Environmental Safety, 70,* 147-153.

Mireji, P. O., Keating, J., Hassanali, A., Impoinvil, D. E., Mbogo, C. M., Muturi, M. N., Nyambaka, H., Kenya E. U., Githure, J. I., & Beier, J. C. (2010a). Expression of metallothionein and alpha-tubulin in heavy metal-tolerant *Anopheles gambiae sensu stricto* (Diptera: Culicidae). *Ecotoxicology and Environmental Safety, 73,* 46-50.

Mireji, P. O., Keating J., Hassanali, A., Mbogo, C. M., Muturi, M. N., Githure, J. I., & Beier, J. C. (2010b). Biological cost of tolerance to heavy metals in the mosquito *Anopheles gambiae. Medical and Veterinary Entomology, 24,* 101-107.

Moreno-Garcia, M., Vargas, V., Ramirez-Bello, I., Hernandez-Maninez, G., & Lanz-Mendoza, H. (2015). Bacterial exposure at the larval stage induced sexual immune dimorphism and priming in adult *Aedes aegypti* mosquitoes. *PLoS One, 10,* e0133240.

Morin, R., Bainbridge, M., Fejes, A., Hirst, M., Krzywinski, M., Pugh, T., McDonald, H., Varhol, R., Jones, S., & Marra, M. (2008). Profiling the Hela S3 transcriptome using randomly primed cDNA and massively parallel short-read sequencing. *Biotechniques, 45,* 81-94.

Mortazavi, A., Wlliams, B. A., McCue, K., Schaeffer, L., & Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods, 5,* 621-628.

Moulder, R., Bhosale, S. D., Goodlett, D. R., & Lahesmaa, R. (2018). Analysis of the plasma proteome using iTRAQ and TMT-based Isobaric labeling. *Mass Spectrum Review, 37,* 583-606.

Moyle, P. B. (1976). *Inland fisheries of California.* University press, Berkely, 10-30.

Mueller, U. G., & Wolfenbarger, L. L. (1999). "AFLP genotyping and fingerprinting". *Trends in Ecological Evolution, 14,* 389-394.

Mukhtar, M., Herrel, N., Amerasinghe, F. P., Ensink, J., van der Hoek, W., & Konradsen, F. (2003). Role of waste water irrigation in mosquito breeding in south Punjab, Pakistan. *Southeast Asian Journal of Tropical Medicine and Public Health, 34,* 72-80.

Munchbach, M., Quadroni, M.; Miotto, G., & James, P. (2000). Quantitation and facilitated *de novo* sequencing of proteins by isotopic N-terminal labeling of peptides with a fragmentation-directing moiety. *Analytical Chemistry, 72,* 40-47.

Mungai, T. M., & Wang, J. (2019). Heavy metal pollution in suburban topsoil of Nyeri, Kapsabet, Voi, Ngong and Juja towns in Kenya. *Springer Nature., https://doi.org/10.1007/s42452-019-0996-0.*

Murphy, J. P., Stapels, M., Fadgen, K., & Germanos, S. (2008). A reproducible method for online RP/RP 2D nano LC/Ms for analysis of proteomic samples. Presented at: 56th ASMS Conference on Mass Spectrometry. CO, USA.

Musasia, F. K., Isaac, A. O., Masiga, D. K., Omedo, I. A., Mwakubambanya, R., Ochieng', R., & Mireji, P. O. (2013). Sex-specific induction of CYP6 cytochrome P450 genes in cadmium and lead tolerant *Anopheles gambiae*. *Malaria Journal, 12,* 97.

Nagalakshmi, U., Wang, Z., Waern, K., Shou, C., Raha, D., Gerstein, M., & Synder, M. (2008). The transcriptional landscape of the yeast whole genome defined by RNA sequencing. *Science, 320,* 1344-1349.

Nagele, E., Vollmer, M., & Horth, P. (2003). Two-dimensional nano-liquid chromatography-mass spectrometry system for applications in proteomics. *Journal of Chromatography A, 1009,* 197-205.

Nakamura, T., Kuromitsu, J., & Oda, Y. (2008). Evaluation of comprehensive multidimensional separations using reversed-phase, reversed-phase liquid chromatography/mass spectrometry for shotgun proteomics. *Journal of Proteome Research, 7*(3), 1007-1011.

Narayanan, S. (1989). *DNA probes. In: Principles and Applications of Laboratory Instrumentation.* Chicago, ASCP Press, 185-191.

Narayanan, S. (1991). Applications of Restriction Fragment Length Polmorphism. *Annals of Clinical and Laboratory Science, 21,* 4.

Nassbaum, R. L., Lesko, J. G., Lewis, R. A., Ledbetter, S. A., & Ledbetter, D. H. (1987). Isolation of anonymous DNA sequences from within a submicroscopic X chromosomal deletion in a patient with choroideremia, deafness and mental retardation. *Proceedings of the National Academy of Science of the United States of America, 84,* 6521-6525.

Nauen, R. (2007). Insecticide resistance in disease vectors of public health importance. *Pest Management Science, 63,* 628-633.

Nchoutpouen, E., Talipouo, A., Djiappi-Tchamen, B., Djamouko-Djonkam, L., Kopya, E., Ngadjeu, C. S., Doumbe-Belisse, P., Awono-Ambene, P., Kekeunou, S., Wondji, C. S., & Antonio-Nkondjio, C. (2019). *Culex* species diversity, susceptibility to insecticides and role as potential vector of Lymphatic filariasis in the city of Yaounde, Cameroon. *PLoS Neglected Tropical Diseases, 13,* e0007229.

Nemeth-Cawley, J. F., Tangarone, B. S., & Rouse, J. C. (2003). "Top Down" characterization is a complimentary technique to peptide sequencing for identifying protein species in complex mixtures. *Journal of Proteome Research, 2,* 495-505.

Nesvizhskii, A. I., & Aebersold, R. (2005). Interpretation of shotgun proteomic data: the protein inference problem. *Molecular and Cellular Proteomics, 4*(10), 1419-1440.

Nicholson, G. M. (2007). Fighting the global pest problem: preface to the special toxicon issue on insecticidal toxins and their potential for insect pest control. *Journal of the International Society on Toxinology, 49*(4), 413-422.

Njunge, J. M., Oyaro, I. N., Kibinge, N. K., Rono, M. K., Kariuki, S. M., Newton, C. R., Berkley, J. A., & Gitau, E. N. (2017). Cerebrospinal fluid markers to distinguish bacterial meningitis from cerebral malaria in children. *Wellcome Trust Open Research, 2,* 47.

Noor, A. M., Mutheu, J. J., Tatem, A. J., Hay, S. J., & Snow, R. W. (2009). Insecticide-treated net coverage in Africa: mapping progress in 2000-2007. *The Lancet, 373,* 58-67.

Nordberg, G. F., Nogawa, K., Nordberg, M., & Friberg, L. (2007). *"*Cadmium*"*. In *Handbook of the Toxicology of Metals.* Pp 445-486, Elsevier.

Norris, D. (2004). Mosquito-borne diseases as a consequence of land use change. *Ecological Health, 1,* 19-24.

Novak, R. J., Gubler, D. J., & Underwood, D. (1985). Evaluation of slow-release formulations of temephos (Abate) and *Bacillus thuringiensis* var. *israelensis* for the control of *Aedes aegypti* in Puerto Rico. *Journal of American Mosquito Control Association, 1*, 449-453.

Novotna, L., Emmerova, T., Horak, D., Kucerova, Z., & Ticha, M. (2010). Iminodiacetic Acid modified Magnetic Poly (2-hydroxyethl Methacrylate)-based Microspheres for Phosphopeptide Enrichment. *Journal of Chromatography A, 1317*(51), 8032-8040.

Nzeve, J. K., Njuguna, S. G. & Kitur, E. C. (2015). Assessment of Heavy Metal contamination in surface Water of Masinga Reservoir, Kenya. *Journal of Natural Sciences Research, 5*(2), 2224-3186

O`Farrell, P. (1975). High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry, 250*(10), 4007-4021.

Ochieng' E. Z., Lalah, J. O. & Wandiga S. O. (2007). Analysis of Heavy Metals in Water and Surface Sediment in five Rift Valley Lakes in Kenya for Assessment of Recent Increase in Anthropogenic Activities. *Bulletin of Environmental Contamination Toxicology, 79,* 570-576.

Oda, Y., Huang, K., Cross, F. R., Cowburn, D., & Chait, B. T. (1999). Accurate quantitation of protein expression and site-specific phosphorylation. *Proceedings of the National Academy of sciences of the United States of America, 96* (12), 6591-6596.

Ohba, S. Y., Kawaia, H., Dida, G. O., Juma, D., Sonye, G., Minakawa, N., & Takagi, M. (2010). Predators of *Anopheles gambiae sensu lato* (Diptera: Culicidae) Larvae in wetlands, Western Kenya: Confirmation by polymerase chain reaction method. *Journal of Medical Entomology, 47,* 783-787.

Ohsugi, T., Nishida, R., & Fukami, H. (1985). Oviposition stimulant of *Papilo xuthus*, a citrus feeding swallow tail butterfly. *Agriculture, Biology and Chemistry, 49,* 1897-1900.

Okogun, G. R. A. (2005). Life-table analysis of *Anopheles* malaria vectors: generational mortality as tool in mosquito vector abundance and control studies. *Journal of VectorBorne Diseases, 42,* 45-53.

Old, W. M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K. G., Mendoza, A., & Sevinsky, J. R. (2005). Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Molecular and Cellular Proteomics, 4*(10), 1487-1502.

Oliver, S. V., & Brooke, B. D. (2016). The role of oxidative stress in the longevity and insecticide resistance phenotype of the major malaria vectors *Anopheles arabiensis* and *Anopheles funestus. PLoS One, 11,* e0151049.

Oliver, S. V., & Brooke, B. D. (2018). The effect of metal pollution on the life history and insecticide resistance phenotype of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). *PLoS ONE, 13*(2), e0192551.

Oljira, K. (2015). Ethnobotanical survey of plants traditionally used for malaria prevention and treatment in selected resettlement and indigenous villages in Sasiga district, western Ethiopia. *Journal of Biology, Agriculture and Healthcare, 5*(11), 2224-3208.

Olsen, J. V., Ong, S. E., & Mann, M. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular and Cellular Proteomics, 3,* 608-614.

Olsen, J. V., Macek, B., Lange, O., Makrov, A., Horning, S., & Mann, M. (2007). Higher energy C-trap dissociation for peptide modification analysis. *Nature Methods, 4,* 709-712.

Omlin, F. X., Carlson, J. C., Ogbunugafor, C. B., & Hassanali, A. (2007). *Anopheles gambiae* exploits the treehole ecosystem in western Kenya: a new urban malaria risk? *Journal of Tropical Medicine and Hygiene, 77,* 264-269.

Ong, S. E., Blagoev, B., Kratchmarova, L., Kristensen, D. B., Steen, H., Pandey, A., & Mann, M. (2002). Stable isotope labelling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Molecular Cellullar Proteomics, 1,* 376-386.

Onyari, J. M., & Wandiga, S. O. (1989). Distribution of Cr, Pb, Cd, Zn, Fe and Mn in Lake Victoria sediments, East Africa. *Bulletin of Environmental Contamination and Toxicology, 42,* 807-8l3.

Opiteck, G. J., Jorgenson, J. W., & Anderegg, R. J. (1997). Two-Dimensional SEC/RPLC Couple to Mass Spectrometry for the Analysis of Peptides. *Analytical Chemistry, 69,* 2283-2291.

Opiteck, G. J., Jorgenson, J. W., Moseley, M. A. III., & Anderegg R. J. (1998a). "Two-Dimensional Microcolumn HPLC Coupled to a single Quadrupole Mass Spectrometer for the Elucidation of Sequence Tags and Peptide Mapping". *Journal of Microcolumn Separations, 10,* 365-375.

Opiteck, G. J., Ramirez, S. M., Jorgenson, J. W., & Moseley, M. A. (1998b). "Comprehensive Two Dimensional HPLC for the Isolation of Over-expressed Proteins and Proteome Mapping". *Analytical Biochemistry, 258,* 349-361.

Patton, W. (2002). Detection technologies in proteome analysis. *Journal of Chromatography B, Analytical Technology of Biomedical Life Sciences, 771*(1-2), 3-31.

Patz, J.A., Graczyk, T. K., Geller, N., & Vittor, A. Y. (2000). Effects of environmental change on emerging parasitic diseases. *International Journal of Parasitology, 30,* 1395-1405.

Penque, D. (2009). Two-dimensional gel electrophoresis and mass spectrometry for biomarker discovery. *Proteomics-Clinical Applications, 3*(2), 155-172.

Persidis, A. (1998). Proteomics-An ambitious drug development platform attempts to link gene sequence to expressed phenotype under various physiological states. *Nature Biotechnology, 16*(4), 393-394.

PMI. (2018). *Malaria Operational Plan Report for the Year, 2018.* USAID-CDC, United States of America.

Poupardin, R., Reynaud, S., & Strode, C. (2008). Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito *Aedes aegypti*: impact on larval tolerance to chemical insecticides. *Insect Biochemistry and Molecular Biology, 38,* 540-551.

Poupardin, R., Riaz, M. A., Vontas, J., David, J. P., & Reynaud, S. (2010). Transcription profiling of eleven cytochrome P450s potentially involved in xenobiotic metabolism in the mosquito *Aedes aegypti*. *Insect Molecular Biology, 19,* 185-193.

Priness, I., Maimon, O., & Ben-gal, I. (2007). Evaluation of gene-expression clustering via mutual information distance measure. *BMC Bioinformatics, 8,* 111.

Protopopoff, N., Verhaeghen, K., van Bortel, W., Roelants, P., Marcotty, T., Baza, D., D'Alessandro, U., & Coosemans, M. (2008). A significant increase in kdr in *Anopheles gambiae* is associated with an intensive vector control intervention in Burundi highlands. *Tropical Medicine International Health, 13,* 1479-1487.

Prugnolle, F., Durand, P., Neel, C., Ollomo, B., Ayala, F. J., Arnathau, C., Etienne, L., Mpoudi-Ngole, E., Nkoghe, D., Leroy, E., Delaporte, E., Peeters, M., & Renaud, F. (2010). African great apes are natural hosts of multiple related malaria species, including *Plasmodium falciparum. Proceedings of the National Academy of Sciences of the United States of America,* Early edition, 1-6.

Pulverer, W., Noehammer, C., Vierlinger, K., & Weinhaeusel, A. (2012*). Principles and Application of Microarray Technology in Thyroid Cancer Research.* Updates in the understanding and management of Thyroid cancer.

Qiao-qiao, H. I., Guang-Wei, Z. H. U., & Langdon, A. (2007). Bioaccumulation of heavy metals in fishes from Taihu Lake, China. *Journal of Environmental Science, 19,* 1500-1504.

Qu, J., Jusko, W. J., & Straubinnger, R. M. (2006). Utility of cleavable isotope-coded affinity-tagged reagents for quantification of low-copy proteins induced by methylprednisolone using liquid chromatography/tandem mass spectrometry. *Analytical Chemistry, 78*(13), 4543-4552.

Ramirez, D. C., & Gimenez, M. S. (2003). Induction of redox changes, inducible nitric oxide synthase and cyclooxygenase-2 by chronic cadmium exposure in mouse peritoneal macrophages. *Toxicology, 145,* 121-132.

Ramos-Fernandez, A., Lopez-Ferrer, D., & Vazquez, J. (2007). Improved method for differential expression proteomics using trypsin-catalyzed ^{18}O labeling with a correction for labeling efficiency. *Molecular and Cellular Proteomics, 6*(7), 1274-1286

Ranson, H., Abdallah, H., & Badolo, A. (2009). "Insecticide resistance in *Anopheles gambiae:* data from the first year of a multi-country study highlight the extent of the problem," *Malaria Journal, 8*(1), 299.

Rawlings, N. D., & Salvesen, G. S. (2013). *Handbook of Proteolytic Enzymes*, Elsevier, San Diego, California, USA.

Raymond, S., & Weintraub, L. (1959). Acrylamide gel as a supporting medium for zone electrophoresis. *Science, 130,* 711.

Rayms-Keller, A., Olson, K. E., McGaw, M., Gray, C., Carlson, J. O., & Beaty, B. J. (1998). Effect of heavy metals on *Aedes aegypti* (Diptera: Culicidae) larvae. *Ecotoxicological and Environmental Safety, 39,* 41-47.

RBM (2015). *Action and investment to defeat malaria 2015-2030.* Geneva: World Health Organization, on behalf of the Roll Back Malaria Partnership Secretariat.

Reed, D. H., Lowe, E. H., Briscoe, D. A., & Frankham, R. (2003). Fitness and adaptation in a novel environment: effect of inbreeding, prior environment and lineage. *Evolution, 57,* 1822-1828.

Rees, J. G., Williams, T. M., Nguli, M. M., Kairu, K. K., & Yobe, A. C. (1996). Contaminant transport and storage in the estuarine creek systems of Mombasa, Kenya. British Geological Survey Overseas Geology Series, *Technical Report* WC/96/ 42.

Reynolds, K. J., Yao, X and Fenselau, C. (2002). Proteolytic ^{18}O labeling for comparative proteomics: evaluation of endoprotease Glu-C as the catalytic agent. *Journal of Proteome Research, 1*(1), 27-33.

Ricardo, S., & Lehmann, R. (2009). An ABC transporter controls export of a *Drosophila* germ cell attractant. *Science, 323,* 943-946.

Riggs, L., Sioma, C., & Regnier, F. E. (2001). Automated signature peptide approach for proteomics. *Journal of Chromatography A, 924,* 359-368.

Righetti, P., & Bossi, A. (1997). Isoelectric focusing in immobilized pH gradients: an update. *Journal of Chromatography B in Biomedical Science Applications, 699*(1-2), 77-89.

Ringwald, P. (2007). Current antimalarial drugs: resistance and new strategies. *Bulletin de l'Academie Nationale de Medecine, 191,* 1273-1284.

Robert, V., Awono-Ambene, H. P., & Thioulouse, J. (1998). Ecology of larval mosquito, with special reference to *Anopheles arabiensis* (Diptera: Culicidae) in market-garden wells in the urban area of Dakar, Senegal. *Journal of Medical Entomology, 35,* 948-955.

Robert, V., Macintyre, K., Keating, J., Trape, J. F., Duchemin, J. B., Warren, M., & Beier, J. C (2003). Malaria transmission in urban sub-Saharan Africa. *American Journal of Tropical Medicine and Hygiene, 68,* 169-176.

Robinson, A. S., Cayol, J. P., & Hendrichs, J. (2002). Recent findings on medfly sexual behavior: implications for SIT. *Florida Entomologist, 85*(1), 171-181.

Roesijadi, G. (1994). Metallothionein induction as a measure of response to metal exposure in aquatic animals. *Environmental Health Perspective, 102,* 91-95.

Rogan, W. J., & Chen, A. (2005). Health risks and benefits of bis (4-chlorophenyl)-1, 1, 1-trichloroethane (DDT). *The Lancet, 366,* 763-773.

Rogerson, S. J., Hviid, L., Duffy, P. E., Leke, R. F., & Taylor, D. W. (2007). Malaria in pregnancy: pathogenesis and immunity. *Lancet Infectious Diseases, 7,* 105-117.

Romi, R., Sabatinelli, G., & Majori, G. (2001). Could malaria reappear in Italy? *Emerging Infectious Diseases, 7*(6), 9-15.

Ross, P. L., Huang,Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha S., Juhasz P., Martin S., Bartlet-jones, M., He, F., Jacobson, A., & Pappin D. J. (2004). Multilexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Molecular and Cellular Proteomics, 3,* 1154-1169.

Rowland, M., Downey, G., Rab, A., Freeman, T., Mohammed, N., Rehman, H., Durrani, A., Reyburn, H., Curtis, C., Lines, J., & Fayaz, M. (2004). DEET mosquito repellent provides personal protection against malaria: A household randomized trial in an Afghan refugee camp in Pakistan. *Tropical Medicine and International Health, 9,* 335-342.

Rozendaal, J. A. (1997). *Vector control-methods for use by individuals and communities*. Geneva: World Health Organization.

Rungrassamee, W., Leelatanawit, R., Jiravanichpaisal, P., Klinbunga, S., & Karoonuthaisiri, N. (2010). Expression and distribution of three heat shock protein genes under heat shock stress and under exposure to *Vibrio harveyi* in *Penaeus monodon*. *Developmental Comparative Immunology, 34,* 1082-1089.

Rupp, H. R. (1996). Adverse assessments of *gambusia affinis*: an alternate view for mosquito control practitioners. *Journal of American Mosquito Control association, 12,* 155-159.

Rydzanicz, K., Lonc, E., & Becker, N. (2009). Current procedures of the integrated urban vector mosquito control as an example in Cotonou (Benin, West Africa) and Wroclaw area (Poland). *Parasitology, 55,* 335-340.

Sachs, J., & Malaney, P. (2002).The economic and social burden of malaria. *Nature, 415,* 680-685.

Sandoval, J., Heyn, H. A., Moran, S., Serra-Musach, J., Pujana, M. A., Bibikova, M., & Esteller, M. (2011). Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics,* 6.

Sargent, T. D., & Dawid, I. B. (1983). Differential gene expression in the gastrula of *Xenopus laevis. Science, 222,* 135-139.

Sarkar, S., Duttagupta, A. K., & Mal, T. K. (2004). Effects of heavy metals on population growth and metallothionein gene expression in the mosquito *Culex quinquefasciatus*, from Calcutta, India. *Environmental Pollution, 127,* 183-193.

Sattler, M. A., Mtasiwa, D., Kiama, M., Premji, Z., Tanner, M., Killeen, G. F., & Lengeler, C. (2005). Habitat characterization and spatial distribution of *Anopheles sp*. mosquito larvae in Dar es Salaam (Tanzania) during an extended dry period. *Malaria Journal, 14,* 4.

Savory, J. J., Kaiser N. K., McKenna, A. M., Xian F., Blankney G. T., Rodgers R. P., Hendrickson C. L., & Marshall, A. G. (2011). Parts-per-billion Fourier transform ion cyclotron resonance mass measurement accuracy with a 'walking' calibration equation. *Analytical Chemistry, 83,* 1732-1736.

Schaferling, M., & Nagl, S. (2006). Optical technologies for the read out and quality control of DNA and protein microarrays. *Analytical and Bioanalytical Chemistry, 385*, 500-517.

Schaper, S. (1999). Evaluation of Costa Rican copepods (Crustacea: Eudecapoda) for larval *Aedes aegypti* control with special reference to *Mesocyclops thermocyclopoides*. *Journal of American Mosquito Control Association, 15,* 510-519.

Schaub, T. M., Hendrickson, C. L., Horning, S., Quinn, J. P., Senko, M. W., & Marshall, A. G. (2008). High-perfomance mass spectrometry: Fourier transform ion cyclotron resonance at 14.5 Tesla. *Analytical Chemistry, 80,* 3985-3990.

Schena, M., Shalon, D., Davis, R. W., & Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science, 270*, 467-470.

Schneider, A. (2000). Triosephosphate isomerase deficiency: historical perspectives and molecular aspects. *Clinical Haematology*, *13*, 119-140.

Schwartz, J. C., Senko, M. W., & Syka, J. E. P. (2002). A two-dimensinal quadrupole ion trap mass spectrometer. *Journal of the American Society for mass Spectrometry, 13*(6), 659-669.

Scott, R. P. W. (1992). Modern Liquid-Chromatography. *Chemical Society Reviews, 21*(2), 137-145.

Sekabira, K., Oryem, O. H., Basamba, T. A., Mutumba, G., & Kakudidi, E. (2010). Assessment of heavy metal pollution in the urban stream sediments and its tributaries. *International Journal of Environmental Science, 7*(3), 435-446.

Service, M. W. (1980). *A guide to medical entomology*. Macmillan Press, London, 24-52.

Service, M. W. (1986*). Lecture Notes on Medical Entomology*. Blackwell Scientific Publications, London, 37-48.

Seyoum, A., Kabiru, E. W., Lwande, W., Killeen, G. F., Hassanali, A., & Knols, B. G. J. (2002a). Repellency of live potted plants against *Anopheles gambiae* from human baits in semi-field experimental huts. *American Journal of Tropical Medicine, 67*, 191-195.

Seyoum, A., Palsson, K., Kung'a, S., Kabiru, E., Lwande, W., Killeen, G. F., Hassanali, A., & Knols, B. G. (2002b). Traditional use of mosquito repellent plants in western Kenya and their evaluation in semi-field experimental huts against *Anopheles gambaie*: ethnobotanical studies and application by thermal expulsion and direct burning. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 96*, 225-231.

Shaalan, E. A., Canyon, D. V., Muller, R., Younes, M. W., Abdel-Wahab, H., & Mansour, A. H. (2007). A mosquito predator survey in townsville, Australia, and an assessment of *Diplonychus sp.* and *Anisops sp.* Predatorial capacity against *Culex annulirostris* mosquito immature. *Journal of Vector Ecology, 32*, 16-21.

Shendure, J. (2008). The beginning of the end for microarrays? *Nature Methods, 5*, 585-587.

Shevchenko, A., Wilm, M., Vorm, O., & Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analytical Chemistry, 68*(5), 850-858.

Shimizu, M., Hochadel, J. F., & Waalkes, M. P. (1997). Effects of glutathione depletion on cadmium-induced metallothionein synthesis, cytotoxicity and proto-oncogene expression in cultured rat myoblasts. *Journal of Toxicology and Environmental Health, 51,* 609-621.

Silva, J. C., Denny, R., Dorschel, C. A., Gorenstein, M., Kass, I. J., & Li, G. Z. (2005). Quantitative proteomic analysis by accurate mass retention time pairs. *Analytical Chemistry, 77*(7), 2187-2200.

Simmonds, M. S. J. (2001). Importance of flavonoids in insect-plant interactions: feeding and oviposition. *Phytochemistry, 56,* 245-252.

Simmonds, M. S. J. (2003). Flavonoid-insect interactions: Recent advances in our knowledge. *Phytochemistry,* 6, 21-30.

Singh, R. K., Dhiman, R. C., & Singh, S. P. (2003). Laboratory studies on the predatory potential of dragon-fly nymphs on mosquito larvae. *Journal of Communicable Diseases, 35,* 96-101.

Singh, H., & Heer, B. K. (2017). Effect of Cadmium chloride on the Development of *Chrysomya megacephala* (Diptera: Calliphoridae) and its importance to postmoterm Interval Estimate. *Journal of Forensic Science & Criminal Investigation, 3*(5), 555-580.

Sinh, N. V., Thi, Yen, N., Minh, D. H., Cong. T. T., Trong, T. V., Hoang L. N., Hoang, S. L., Le L. L., Que, H. V. T., & Kim, K. L. H. (2012). Community-based control of *Aedes aegypti* by using Mesocyclops in Southern Vietnam. *American Journal of Tropical Medicine and Hygiene, 86,* 850-859.

Sinka, M., Bangs, M., Manguin, S., Coetzee, M., Mbogo, C., & Hemingway. (2010). The dorminant *Anopheles* vectors of human malaria in Africa, Europe and Middle East: occurrence data, distribution maps and bionomic precision. *Parasite Vectors, 3*(1), 117.

Sinka, M. E., Bangs, M. J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., & Coetzee, M. (2012). A global map of dominant malaria vectors. *Parasites and Vectors, 5*(1), 69.

Sinkins, S. P., & Gould, F. (2006). Gene drive systems for insect disease vectors. *Nature Review of Genetics, 7,* 427-435.

Siuzdak, G. (2004). *An introduction to mass spectrometry ionization: An excerpt from the Expanding Role of Mass Spectrometry in Biotechnology,* 2nd ed.; MCC press: San Diego, 2005. *Journal of the Association for Laboratory Automation,* 9:50-63.

Smith, R. D., Loo, J. A., Loo, R. R. O., Busman, M., & Udseth, H. R. (1991). Principles and practice of electrospray ionization-mass spectrometry for large polpeptides and proteins. *Mass spectrum Review, 10,* 359-452.

Smith, L. M., & Kelleher, N. L. (2013). Proteoform: A single term describing protein complexity. *Nature Methods, 10,* 186-187.

Smolka, M. B., Zhou, H., Purkayastha, S., & Aebersold, R. (2001). Optimization of the isotope-coded affinity tag-labelling procedure for quantitative proteome analysis. *Analytical Biochemistry, 297,* 25-31.

Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y., & Hay, S. I. (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature, 434,* 214-217.

Soumare, M. K., & Cilek, J. E. (2011). The effectiveness of *Mesocyclops longisetus* (Copepoda) for the control of container-inhabiting mosquitoes in residential environments. *Journal of American Mosquito Control association, 27,* 376-383.

Southon, A., Burke, R., & Camakaris, J. (2013). What can flies tell us about copper homeostasis? *Metallomics, 5,* 1346-1356.

Stafford, G. C., Kelley, P. E., Syka, J. E. P., Reynolds, W. E., & Todd, J. F. J. (1984). Recent improvements in analytical applications of advanced ion-trap technology. *International Journal of Mass Spectrometry Ion Process, 60*(85-98), 85-98.

Steffan, W. A., & Evenhuis, N. L. (1981). Biology of Toxorhynchites. *Annual Review of Entomology, 26,* 159-181.

Stohs, S. J., Bagchi, D., Hassoun, E., & Bagchi, D. (2001). Oxidative mechanisms in the toxicity of chromium and cadmium ions. *Journal of Environmental Pathology Toxicology and Oncology, 20,* 77-88.

Strode, C., Steen, K., Ortelli, F., & Ranson, H. (2006). Differential expression of the detoxification genes in the different life stages of the malaria vector *Anopheles gambiae*. *Insect Molecular Bio1ogy, 15,* 523-530.

Subramaniam, J., Murugan, K., Panneerselvam, C., Kovendan, K., madhiyazhagan, P., Kumar, P. M., Dinesh, D., Chandramohan, B., Suresh, U., & Nicoletti, M. (2015). Eco-friendly control of malaria and arbovirus vectors using mosquito fish *Gambusia affins* and ultra-low dosages of *Mimusops elengi*-synthesized silver nanoparticles: Towards an

intergrative approach? *Environmental Science of Pollution Research International, 22,* 20067-20083.

Suzuki, K. T., Sunaga, H., Aoki, Y., Hatakeyama, S., Sugaya, Y., Sumi, Y., & Suzuki, T. (1988). Binding of cadmium and copper in the mayfly *Baetis thermicus* larvae that inhabit a river polluted with heavy metals. *Comparative Biochemistry and Physiology, 91,* 487-492.

Switzar, L., Giera, M., & Niessen, W. M. A. (2013). Protein digestion: an overview of the available techniques and recent developments. *Journal of Proteome Research, 12*(3), 1067-1077.

Syka, J. E. P., Coon, J. J., Schroeder, M. J., Shabanowitz, J., & Hunt, D. F. (2004). Peptide and protein sequence analysis by election transfer dissociation mass spectrometry. *Proceedings of the National Academy of Science of the United States of America, 101,* 9528-9533.

Szklarczyk, D., Franceschini, A., Kuhn, M., Simonovic, M., Roth, A., Minguez, P., Doerks, T., Stark, M., Muller, J., & Bork, P. (2011). The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Research, 39,* D561-D568.

Tabb, D. L., Wang, X., Carr, S. A., Clauser, K. R., Mertins, P., Chambers, M. C., & Liebler, D. C. (2016). Reproducibility of Differential Proteomic Technologies in CPTAC Fractionated Xenografts. *Journal of Proteome Research*, *15*(3), 691-706.

Tanaka, K. (1998). Proteasomes: Structure and Biology. *Journal of Biochemistry*, *123,* 195-204.

Tangena, J. A., Thammavong, P., Chonephetsarath, S., Logan, J. G., Brey, P. T., & Lindsay, S. W. (2018). Field evaluation of personal protection methods against outdoor-biting mosquitoes in Lao PDR. *Parasites & Vectors, 11,* 661.

Tawatsin, A., Thavara, U., Chansang, U., Chavalittumrong, P., Boonruad, T., Wongsinkongman P., Bansidhi J., & Mulla, M. S. (2006). Field evaluation of DEET, repel care®, and three plant-based essential oil repellents against mosquitoes, black flies (Diptera: Simullidae), and land leeches (Arthynchobdellida: Haemadipsidae) in Thailand," *Journal of the American Mosquito Control Association, 22*(2), 306-313.

Thirwell, C., Eymard, M., Feber, A., Teschendorff, A., Pearce, K., Lechner, M., Widschwendter, M., & Beck, S. (2010). Genome-Wide DNA methylation analysis of archival formalin-

fixed paraffin-fixed paraffin-embedded tissue using the Illumina Infinium Human Methylation 27 bead Chip. *Methods, 52*, 248-254.

Thompson, A., Schafer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., & Hamon, C. (2003). Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Analytical Chemistry*, *75*(8), 1895-1904.

Tiwary, M., Naik, S.N., Tewary, D. K., Mittal, P. K., & Yadav, S. (2007). Chemical composition and larvicidal activities of the essential oil of *Zanthoxylum armatum D C* (Rutaceae) against three mosquito vectors. *Journal of Vector Borne Diseases, 44*(3), 198-204.

Toll, H., Oberacher, H., Swart, R., & Huber, C. G. (2005). Separation, detection and identification of peptides by ion-pair reversed-phase high-perfomance liquid chromatography electrospray ionization mass spectrometry at high and low pH. *Journal of Chromatography A, 1079*(1-2), 274-286.

Toure`, Y. T. V., Petrarca, S. F., Traore´, A., Coulibaly, H. M., Maiga, O., Sankare´, M., Sow, M. A., Di Deco., & Coluzzi, M. (1998). The distribution and inversion polymorphism of chromosomally recognized taxa of the *Anopheles gambiae* complex in Mali, West Africa. *Parasitologia, 40,* 477-511.

Trape, J. F., Zoulani, A. (1987). Malaria and urbanization in central Africa: The example of Brazzaville. Part II: Results of entomological surveys and epidemiological analysis. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 81*, 10-18.

Tsuchihara, K., Suzuki, Y., Wakaguri, H., Irie, T., Tanimoto, K., Hashimoto, S. L., Matsushima, K., Sugano, J. M., Yamashita, R., Nakai, K., Bentley, D., Esumi, H., & Sugano, S. (2009). Massive transcriptional start site analysis of human genes in hypoxia cells. *Nucleic Acid Research, 37*, 2249-2263.

Turelli, M. (2010). Cytoplasmic incompatibility in populations with overlapping generations. *Evolution, 64*, 232-241.

United Nations. (1990*). Human Development Report*: Oxford University Press

United Nations. (1991). *World Urbanization Prospects*, New York.

United Nations. (2003). *World Urbanization Prospects*: *The 2007 revision*. New York: United Nations: Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat.

United Nations. (2004). *World urbanization prospects*: *The 2003 revision.* New York: Upper Saddle River.

UNPF. (1993). *The State of the World,* United Nations Population Fund

UN-Water. (2006). *Kenya National Water Development Report.* Case Study: Kenya 2nd UN World Water Development Report.

Urasa, I. T., & Onyari, J. M. (1986). Element content of lakes in the great Rift Valley of East Africa *1n: 43rd Joint Annual Meeting of the National Institute of Science BKK/BSP. National Convention March 26-29, Norfolk, Virginia.*

Vache, C., Camares, O., Cardoso-Ferreira, M. C., & Dastugue, B. (2007). A potential genomic biomarker for the detection of polycyclic aromatic hydrocarbon pollutants: multidrug resistance gene 49 in *Drosophila melanogaster*. *Environmental Toxicology and Chemistry, 26,* 1418-1424.

van Dam, A. R., & Walton, W. E. (2007). Comparison of mosquito control provided by the Arroyo chub (*Gila orcutti)* and the mosquito fish *(Gambusia affins). Journal of American Mosquito Control Association, 23,* 430-441.

van Straalen, N. M., & Roelofs, D. (2005). Cadmium tolerance in a soil arthropod-a model of real-time microevolution. *Entomologische Berichten, 65,* 105-111.

Varshavsky, A. (1997). The N-end rule pathway of protein degradation. *Genes Cells, 2,* 13-28.

Venkatesan, P., & Jeyachandra, C. M. (1985). Estimation of mosquito predation by water bug *Diplonychus indicus* Venkatesan and Rao. *Indian Journal of Experimental Biology, 23,* 227-229.

Vijayalakshmi, M. A. (1996). Histidine ligand affinity chromatography. *Molecular Biotechnology, 6,* 347-357.

Vittor, A. Y., Gilman, R. H., Tielsch, J., Glass, G., Shields, T., Lozano, W. S., Pinedo-Cancino, V., & Patz, I. A. (2006). The effect of deforestation on the human-biting rate of *Anopheles darlingi,* the primary vector of *falciparum* malaria in the Peruvian, Amazon. *American Journal of Tropical Medicine Hygiene, 74*(1), 3-11.

Vizcaino, J. A., Deutsch, E. W., Wang, R., Csordas, A., Reisinger, F., Rios, D., Dianes, J. A., Sun, Z., Farrah, T., & Bandeira, N. (2014). ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nature Biotechnology, 32,* 223-226.

Vos, P., Hogers, R., & Bleeker, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research, 23*(21), 4407-4414.

Vreysen, M. J. B., Saleh, K. M., Ali, M. Y., Abdulla, A. M., Zhu, Z. R., Juma, K. G., Dyck, V. A., Msangi, A. R., Mkonyi, P. A., & Feldmann, H. U. (2000). *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. *Journal of Economic Entomology, 93,* 123-135.

Vu, S. N., Nguyen, T. Y., Tran,V. P., Truong, U. N., Le, Q. M., Le, V. L., Le, T. N., Bektas, A., Briscomode, A., & Aoskov, J. G. (2005). Elimination of dengue by community programs using Mesocyclops (Copepoda) against *Aedes aegypti* in Central Vietnam. *American Journal of Tropical Medicine and Hygiene, 72,* 67-73.

Vu, S. N., Nguyen, T.Y., Kay, B.H., Marten, G.G., & Reid, J. W. (1998). Eradication of *Aedes aegypti* from a village in Vietnam, using copepods and community participation. *American Journal of Tropical Medicine and Hygiene, 59,* 657-660.

Walker, K., & Lynch, M. (2007). Contributions of *Anopheles* larval control to malaria suppression in tropical Africa: review of achievements and potential. *Medical and Veterinary Entomology, 21,* 2-21.

Wall, D. B., Berger, S. J., Finch, J. W., Cohen, S. A., Richardson, K., Chapman, R., Drabble, D., Brown, J., & Gostick, D. (2002). Continous sample depositions from reversed phase liquid chromatography to tracks on a matrix-assisted laser desorption/ionization precoated target for the analysis of protein digests. *Electrophoresis, 23,* 3193-3204.

Walton, W. E. (2007). Larvivorous fish including *Gambusia. Journal of American Mosquito Control Association, 23,* 184-220.

Wandiga, S. O., & Onyari, J. M. (1987). The concentration of heavy metals: Manganese, iron, copper, zinc, cadmium and lead in sediments and fish from the Winam Gulf of Lake Victoria and fish bought in Mombasa town markets. *Kenyan Journal of Science, 8,* 5-18.

Wang, Z., & Brown, D. D. (1991). A gene expression screen. *Proceedings of the National Academy of Science of the United States of America, 88,* 11505-11509.

Wang, S. J., Lengeler, C., Smith, T. A., Vounatsou, P., & Cisse, G. (2005). Rapid urban malaria appraisal (RUMA) in sub-Saharan Africa. *Malaria Journal, 4,* 40.

Wang, F., Ye, M., & Dong, J. (2008a). Improvement of performance in label-free quantitative proteome analysis ith monolith electrospray ionization emitter. *Journal of Separation Science, 31*(14), 2589-2597.

Wang, M., You, J., Bemis, K. G., Tegeler, T. J., & Brown, D. P. G. (2008b). Label-free mass spectrometry based protein quantification technologies in proteomic analysis. *Briefings in Functional Genomics and Proteomics, 7*(5), 329-339.

Wang, Z., Gertein, M., & Synder, M. (2009). RNASeq: A revolutionary tool for transcriptomics. *Nature Review of Genetics, 10,* 57-63.

Wang, N., & Li, L. (2010). Reproducible Microwave-assisted Acid Hydrolysis of Proteins using a household Microwave Oven and its Combination with LC-ESI MS=MS for Mapping Protein Sequences and Modifications. *Journal of the American Society for Mass Spectrometry, 21*(9), 1573-1587.

Warrell, D. A., Molyneux, M. E., & and Beales P. F. (1990). Severe and complicated malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 84,* 1-65.

Washburn, M. P., Wolters, D., & Yates, J. R. (2001). Large-Scale Analysis of the Yeast proteome by Multidimensional Protein Identification Technology. *Nature Biotechnology, 19*(3), 242-247.

Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M., Harris, R., Williams, K., & HumpherySmith, I. (1995). Progress with gene-product mapping of the mollicutes: *Mycoplasma genitalium*. *Electrophoresis, 16*(7), 1090-1094.

Waters, E. R., Aevermann, B. D., & Sanders-Reed, Z. (2008). Comparative analysis of the small heat shock proteins in three angiosperm genomes. *Cell Stress Chaperones, 13,* 127-142.

Webber, B. L., Raghu, S., & Edwards, O. R. (2015). Opinion: is CRISPR-based gene drive a biocontrol silver bullet or global conservation threat? *Proceedings of the National Academy of Science of the United States of America, 112,* 10565-10567.

Wedepohl, K. H. (1995). "The composition of the continental crust," geochimmica et Cosmochimica. *Acta, 59*(7), 1217-1232.

Weiss, W., & Gorg, A. (2009). High-resolution two dimensional electrophoresis. *Methods in Molecular Biology, 564,* 13-32.

Weka, M., Hopkins, R. J., & Curtis, C. (2004). Ethnobotanical survey and testing of plants traditionally used against hematophagous insects in Eritrea. *Journal of ethnopharmacology*, *95*, 95-101.

Wentsel, R., McIntosh, A., & Atchinson, G. (1978). Evidence of resistance to metals in larvae of the midge *Chironomus tentans* in a metal contaminated lake. *Bulletin of Environmental Contamination and Toxicology*, *20*, 451-455.

Westman-Brinkmalm, A., Abramsson, A., & Pannee, J. (2011). SILAC zebrafish for quantitative analysis of protein turnover and tissue regeneration. *Journal of proteomics*, *75*(2), 425-434.

Weston, A. & Brown, P. R. (1997). *HPLC and CE-Principles and Practice.* Academic press, San Diego, CA, USA.

Wheelan, S. J., Martinez, M. F., & Boeke, J. D. (2008). The incredible shrinking world of DNA microarrays. *Molecular Biosystems*, *4*, 726-732.

White, G. B. (1974). *Anopheles gambiae* complex and disease transmission in Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *68*, 278-302.

White, B. J., Collins, F. H., & Besansky, N. J. (2011). Evolution of *Anopheles gambiae* in Relation to Humans and Malaria. *Annual Review of Ecology, Evolution and Systematics*, *42*, 111-132.

Whitelegge, J. P., Zhang, H., Aguilera, R., Taylor, R. M., & Cramer, W. A. (2002). Full sub-unit coverage liquid chromatography electrospray ionization mass spectrometry (LCMS+) of an oligomeric membrane protein: cytochrome b(6)f complex from spinach and the *Cyanobacterium mastogocladus laminosus*. *Molecular and Cellular Proteomics*, *1*, 816-827.

Whitten, M. M., Tew, I. F., Lee, B. L., & Ratcliffe, N. A. (2004). A novel role for an insect apolipoprotein (apolipophorin III) in beta-1, 3-glucan pattern recognition and cellular encapsulation reactions. *Journal of Immunology*, *172*, 2177-2185.

WHO. (1992a). *Cadmium.* International Programme on Chemical safety. Environmental Health Criterial 134, 156. http://www.inchem.org/documents/ehc/ehc134.htm).

WHO. (1992b). *Cadmium-Environmental aspects*. International Programme on Chemical Safety Environmental Health Criteria 135. http://www.inchem.org/documents/ehc/ehc/ehc135.htm).

WHO. (1993). *Intergrated vector control. Seventh report of the WHO expert committee on vector biology and control.*Technical report series, no. 688.

WHO. (2006). *Position statement: Indoor residual spraying-Use of indoor residual spraying for scaling up global malaria control and elimination.* World Health Organization.

WHO. (2007). *Health risks of heavy metals from long-range transboundary air pollution.* Copenhagen, World Health Organization Regional Office for Europe (http://www.euro.who.int/document/E91044.pdf).

WHO. (2010). *Public Health and Environment*. Avenue Appia, 1211 Geneva 27, Switzerland.

WHO. (2011a). *Global Insecticide Use for Vector-Borne Disease Control- A 10-Year Assessment (2000-2009).* World Health Organization.

WHO. (2011b).*World Malaria Report 2011*.World Health Organization.

WHO. (2013).*World Malaria Report 2013.* WHO Global Malaria Programme.

WHO. (2014). *World Malaria Report*. Geneva: World Health Organization.

WHO. (2015). *Global Technical Strategy for Malaria 2016-203*0. Geneva: World Health Organization.

WHO. (2016). *Malaria Fact Sheet,* World Health Organization, Geneva, Switzerland.

WHO. (2018). *World Malaria Report*. Geneva: World Health Organization.

Wieland, L., Bolger, G., Asouline, G., & Wigler, M. (1990). A method for differenial cloning; Gene amplification following subtractive hybridization. *Proceedings of the National Academy of Science of the United States of America, 87,* 2720-2724.

Wienkoop, S., Larrainzar, E., Niemann, M., Gonzalez, E. M., Lehmann, U., & Weckwerth, W. (2006). Stable isotope-free quantitative shotgun proteomics combined with sample pattern recognition for rapid diagnostics. *Journal of Separation Science, 29*(18), 2793-2801.

Wigglesworth, V. B. (1976). *Insects and the life of man*. John Wiley and Sons Inc; New York. 95-102.

Wilczek, G., babczynska, A., Augustyniak, M., & Migula, P. (2004). Relations between metals (Zn, Pb, Cd and Cu) and glutathione-dependent detoxifying enzymnes in spiders from heavy metal pollution gradient. *Environmental Pollution, 132,* 453-461.

Wilhelm, B. T., Marguerat, S., Watt, S., Schubert, F., Wood, V., Goodhead, I., Penkett, C. J., Rogers, J., & Baheler, J. (2008). Dynamic repertoire of a eukaryotic transcriptome surveyed at single nucleotide resolution. *Nature, 453,* 1239-1243.

Wilke, A. B. B., Nimmo, D. D., John, O., Kojin, B. B., Capurro, M. L., & Marrelli, M. T. (2009). Mini-review: Genetic enhancements to the sterile insect technique to control mosquito populations. *Asia Pacific Journal of Molecular Biology and Biotechnology, 17*(3), 65-74.

Wilke, A. B. B., & Marrelli, T. M. (2012). Genetic control of mosquitoes: population suppression strategies. *Revista do Instituto de Medicina Tropical de São de Paulo, 54*(5), 287-292.

Wilkins, M., Sanchez, J., Gooley, A., Appel, R., HumpherySmith, I., Hochstrasser, D., & Williams, K. (1996). Progress with proteome projects: why all proteins expessed by a genome should be identified and how to do it. *Biotechnology and Genetic Engineering Review, 13,* 19-50.

Wilkinson, J. M. (1986). *Fragmentation of polypeptides by enzymatic methods: Practical protein chemistry.* John Wiley and Sons, New York.

Wolters, D. A., Washburn, M. P., & Yates, J. R. (2001). An automated multidimensional protein identification technology for shotgun proteomics. *Analytical Chemistry, 73*(23), 5683-5690.

Wu, J., Lenchik, N., Pabst, M., Solomon, S., Shull, J., & Gerling, I. (2005). Functional characterization of two-dimensional gel-separated proteins using sequential staining. *Electrophoresis, 26*(1), 225-237.

Wunschel, D., Tolic, L. P., Feng, B., & Smith, R. D. (2000). Electrospray ionization fourier transform ion cyclotron resonance analysis of large polymerase chain reaction products. *Journal of American Society of Mass Spectrometry, 11,* 333-337.

Xie, F., Liu, T., Qian, W. J., Petyuk, V. A., & Smith, R. D. (2011). Liquid chromatography-Mass spectrometry-based Quantitatve proteomics. *Journal of Biological Chemistry, 286*(29), 25443-25449.

Xu, Y. T., Wang, J., Yin, R., Qiu, M. T., Xu, L., Wang, J., & Xu, L. (2014). Genetic polymorphisms in Glutathione S-transferase Omega (GSTO) and cancer risk: a meta-analysis of 20 studies. *Science of Reproduction, 4,* 65-78.

Yadouleton, A., Asidi, A., & Djouaka, R. (2009). Development of vegetable farming: a cause of the emergence of insecticide resistance in populations of *Anopheles gambiae* in urban areas of Benin. *Malaria Journal, 8,* 103.

Yan, W. & Chen, S. S. (2005). Mass spectrometry based quantitative proteomic profiling. *Briefings in Functional Genomics and Proteomics, 4*(1), 27-38.

Yang, H., Wang, Y., & Xiao, Y. (2011). A bi-functional anti-thrombosis protein containing both direct-acting fibrinogenolytic and plasminogen-activating activities. *PloS one, 6,* e17519.

Yao, X., Freas, A., Ramirez, J., Demirev, P. A., & Fensalau, C. (2001). Proteolytic ^{18}O labelling for comparative proteomics: model studies with two serotypes of adenovirus. *Analytical Chemistry, 73*(13), 2836-2842.

Yap, H. H. (1986). Effectiveness of soap formulations containing DEET and permethrin as personal protection against outdoor mosquitoes in Malaysia. *Journal of American Mosquito control Association, 2,* 63-67.

Yap, H., Jahangir, K., & Zairi, J. (2000). Field efficacy of four insect repellent products against vector mosquitoes in a tropical environment. *Journal of the American Mosquito Control Association, 16,* 241-244.

Yates, J. R., Ruse, C. I., & Nakorchevsky, A. (2009). ^{18}O stable isotope labeling in MS-based proteomics. *Briefings in Functional Genomics and Proteomics, 8*(2), 136-144.

Ye, K., Jin, S., Ataai, M. M., Schultz, J. S., & Ibeh, J. (2004). Tagging retrovirus vectors with a metal binding peptide and one-step purification by immobilized metal affinity chromatography. *Journal of Virology, 78,* 9820-9827.

Ye, X. L. B., Anderson, T., & Blonder, J. (2009). ^{18}O stable isotope labeling in MS-based proteeomics. *Briefings in Functional Genomics and Proteomics, 8*(2), 136-144.

Yin, N. G. S., Abdullah, S., & Phin, C. K. (2013). Phytochemical consituents from leaves of *Elaeis guineensis* and their antioxidant and antimicrobial activities. *International Journal of Pharmacy and Pharmaceutical Sciences, 4,* 137-140.

Ying, S. H., & Feng, M. G. (2011). A conidial protein (CPI5) of *Beauveria bassiana* contributes to the conidial tolerance of the entomopathogenic fungus to thermal and oxidative stresses. *Applied Microbiology and Biotechnology, 90,* 1711-1720.

Youmsi, R. D. F., Fokou, P. V. T., Menkem, E. Z., Bakarnga-Via, L., Keumoe, R., Nana, V., & Boyom, F. F. (2017). Ethnobotanical survey of medicinal plants used as insects repellents

in six malaria endemic localities of Cameroon. *Journal of Ethnobiology and Ethnomedicine, 13,* 33.

Zachariou, M. (2004). Immobilized metal ion affinity chromatography of proteins.*Methods in Molecular Biology, 251,* 89-102.

Zaim, M., Aitio, A., & Nakashima, N. (2000). Safety of pyrethroid treated mosquito nets. *Medical Veterinary and Entomology, 14,* 1-5.

Zeigler, A., Donald, J., Brunn S. D., & Williams, J. F. (2003). *World urban development*. In Brunn, Stanley D., Jack F. Williams and Donald J. Zeigler (Eds), *Cities of the world: World regional development.* Lanham: Rowman and Littlefield, 1-46.

Zhang, Y., Fonslow, B. R., Shan, B., Baek, M. C., & Yates, J. R. (2013). Protein analysis by shotgun/bottom-up proteomics. *Chemistry Review, 113,* 2343-2394.

Zhao, L., & Jones, W. A. (2012). Expression of heat shock protein genes in insect stress responses. *Minireview, 9,* 93-101.

Zhen, Y., Xu, N., Richardson, B., Becklin, R., Savage, J. R., Blake, K., & Peltier, J. M. (2004). Development of an LC-MALDI method for the analysis of protein complexes. *Journal of American Society of Mass Spectrometry, 15,* 803-882.

Zhou, H., Dai, J., & Sheng, Q. H. (2007). A fully automated 2D LC-MS method utilizing online continuous pH and RP gradients for global proteome analysis. *Electrophoresis, 28*(23), 4311-4319.

Zhou, H., Low, T. Y., Henrich, M. L., van der Toorn, H., Schwend, T., Zou, H., Mohammed, S., & Heck, A. J. R. (2013). Enhancing the Identification of Phosphopeptides from putative Basophilic Kinase Substrates using Ti (IV) Based IMAC enrichment. *Molecular and Cellular proteomics, 12*(9), 2673.

Zhu, L., Muller, G. C., Marshall, J. M., Arheart, K. L., Qualls, W. A., Hlaing, W. M., Schlein, Y., Traore, S. F., Doumbia, S., & Beier, J. C. (2017). Is outdoor vector control needed for malaria elimination? An individual-based modelling study. *Malaria Journal, 16,* 266.

Zimmy-Arndt, U., Schmid, M., Ackermann, R., & Jungblut, P. (2009). Classical Proteomics: two dimensional electrophoresis/MALDI mass spectrometry. *Methods in Molecular Biology, 492,* 65-91.

Zou, Z., Picheng, Z., Weng, H., Mita, K., & Jiang, H. (2009). A comparative analysis of serpin genes in the silkworm genome. *Genomics, 93,* 367-375.

Zubarev, R. A., Kelleher, N. L., & McLafferty, F. W. (1998). Electron capture dissociation of multiply charged protein cations. A non-ergodic process. *Journal of American Chemical Society, 120,* 3265-3266.

Zug, R., & Hammerstein, P. (2012). Still a host of hosts for *Wolbachia:* analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PloS ONE, 7,* e38544.

Zvinowanda, C. M., Okonkwo, J. O., Shabalala, P. N., & Agyei, N. M. (2009). A novel adsorbent for heavy metal remediation in aqueous environments. *International Journal of Environmental Science, 3,* 425-434.

APPENDICES

Appendix A. Total proteins identified from *An. gambiae s. s* L3 larvae

Total proteins in Cadmium group	Total proteins in control group
AGAP000005-PA	AGAP002355-PB
AGAP000044-PA	AGAP002363-PA
AGAP000167-PA	AGAP002374-PA
AGAP000170-PA	AGAP002387-PA
AGAP000180-PA	AGAP002390-PA
AGAP000235-PC	AGAP002395-PA
AGAP000260-PC	AGAP002399-PA
AGAP000261-PA	AGAP002401-PA
AGAP000270-PA	AGAP002407-PA
AGAP000272-PA	AGAP002413-PD
AGAP000278-PA	AGAP002414-PA
AGAP000291-PA	AGAP002422-PA
AGAP000297-PA	AGAP002437-PA
AGAP000305-PA	AGAP002440-PC
AGAP000306-PA	AGAP002456-PA
AGAP000308-PA	AGAP002457-PA
AGAP000315-PA	AGAP002464-PA
AGAP000344-PB	AGAP002465-PA
AGAP000352-PA	AGAP002468-PA
AGAP000359-PA	AGAP002470-PA
AGAP000375-PA	AGAP002473-PA
AGAP000399-PA	AGAP002477-PA
AGAP000403-PA	AGAP002491-PA
AGAP000415-PA	AGAP002499-PA
AGAP000416-PC	AGAP002500-PA
AGAP000437-PA	AGAP002521-PB

AGAP000462-PB
AGAP000508-PA
AGAP000526-PA
AGAP000536-PA
AGAP000538-PA
AGAP000541-PB
AGAP000550-PA
AGAP000572-PA
AGAP000573-PB
AGAP000615-PA
AGAP000622-PB
AGAP000625-PA
AGAP000626-PA
AGAP000651-PC
AGAP000654-PA
AGAP000655-PA
AGAP000672-PB
AGAP002440-PC
AGAP002684-PB
AGAP002777-PA
AGAP002788-PA
AGAP003154-PB
AGAP003168-PA
AGAP003200-PA
AGAP003219-PA
AGAP003331-PB
AGAP003334-PA
AGAP003476-PA
AGAP003582-PA
AGAP003686-PA
AGAP003719-PA
AGAP002534-PB
AGAP002557-PA
AGAP002559-PB
AGAP002564-PE
AGAP002568-PA
AGAP002592-PA
AGAP002593-PA
AGAP002596-PA
AGAP002612-PA
AGAP002613-PA
AGAP002619-PA
AGAP002645-PA
AGAP002654-PA
AGAP002661-PA
AGAP002667-PA
AGAP002684-PB
AGAP002726-PA
AGAP002728-PA
AGAP002739-PA
AGAP002743-PA
AGAP002754-PA
AGAP002777-PA
AGAP002782-PA
AGAP002788-PA
AGAP002812-PA
AGAP002813-PA
AGAP002849-PA
AGAP002850-PA
AGAP002878-PA
AGAP002879-PA
AGAP002889-PA

AGAP003834-PA
AGAP003883-PA
AGAP003937-PA
AGAP004002-PA
AGAP004031-PA
AGAP004093-PA
AGAP004110-PA
AGAP004146-PA
AGAP004212-PA
AGAP004321-PA
AGAP004362-PB
AGAP004407-PA
AGAP004455-PA
AGAP004462-PA
AGAP004484-PA
AGAP004533-PA
AGAP004566-PA
AGAP004625-PB
AGAP004638-PA
AGAP004723-PA
AGAP004743-PA
AGAP004845-PB
AGAP004886-PB
AGAP004919-PA
AGAP004975-PA
AGAP004996-PA
AGAP005003-PA
AGAP005015-PA
AGAP005032-PB
AGAP005076-PC
AGAP005081-PA
AGAP002892-PA
AGAP002919-PA
AGAP002921-PB
AGAP002925-PC
AGAP002948-PA
AGAP003007-PA
AGAP003008-PB
AGAP003016-PA
AGAP003025-PC
AGAP003043-PB
AGAP003048-PA
AGAP003052-PA
AGAP003057-PA
AGAP003069-PA
AGAP003091-PA
AGAP003095-PA
AGAP003119-PA
AGAP003134-PA
AGAP003136-PA
AGAP003141-PB
AGAP003154-PB
AGAP003168-PA
AGAP003173-PA
AGAP003178-PA
AGAP003200-PA
AGAP003212-PA
AGAP003216-PA
AGAP003219-PA
AGAP003229-PA
AGAP003238-PC
AGAP003246-PA

AGAP005095-PA	AGAP003250-PA
AGAP005134-PA	AGAP003277-PD
AGAP005197-PA	AGAP003289-PA
AGAP005234-PB	AGAP003308-PA
AGAP005316-PA	AGAP003331-PB
AGAP005327-PA	AGAP003334-PA
AGAP005402-PA	AGAP003337-PA
AGAP005427-PA	AGAP003360-PA
AGAP005462-PA	AGAP003390-PA
AGAP005486-PA	AGAP003398-PF
AGAP005559-PA	AGAP003414-PB
AGAP005569-PA	AGAP003462-PA
AGAP005581-PA	AGAP003476-PA
AGAP005603-PA	AGAP003486-PA
AGAP005609-PB	AGAP003507-PA
AGAP005615-PA	AGAP003521-PA
AGAP005618-PA	AGAP003532-PA
AGAP005627-PD	AGAP003538-PA
AGAP005641-PA	AGAP003541-PC
AGAP005644-PA	AGAP003553-PA
AGAP005663-PA	AGAP003556-PA
AGAP005671-PA	AGAP003578-PA
AGAP005685-PA	AGAP003581-PA
AGAP005693-PA	AGAP003582-PA
AGAP005695-PA	AGAP003592-PB
AGAP005696-PA	AGAP003598-PA
AGAP005723-PA	AGAP003612-PA
AGAP005739-PA	AGAP003625-PA
AGAP005766-PA	AGAP003626-PA
AGAP005813-PA	AGAP003640-PA
AGAP005838-PA	AGAP003663-PA

AGAP005858-PA	AGAP003686-PA
AGAP005861-PB	AGAP003689-PA
AGAP005914-PA	AGAP003701-PA
AGAP005947-PA	AGAP003719-PA
AGAP005960-PA	AGAP003742-PB
AGAP005979-PA	AGAP003768-PB
AGAP005981-PA	AGAP003770-PA
AGAP005991-PA	AGAP003790-PC
AGAP005996-PA	AGAP003807-PA
AGAP005997-PA	AGAP003816-PA
AGAP005998-PA	AGAP003834-PA
AGAP006009-PA	AGAP003870-PA
AGAP006012-PA	AGAP003878-PA
AGAP006037-PA	AGAP003879-PA
AGAP006070-PA	AGAP003883-PA
AGAP006090-PA	AGAP003919-PA
AGAP006095-PA	AGAP003935-PA
AGAP006102-PA	AGAP003937-PA
AGAP006103-PA	AGAP003968-PA
AGAP006145-PA	AGAP003971-PA
AGAP006146-PA	AGAP003987-PA
AGAP006208-PA	AGAP004002-PA
AGAP006209-PA	AGAP004015-PA
AGAP006275-PA	AGAP004031-PA
AGAP006278-PA	AGAP004064-PB
AGAP006283-PB	AGAP004065-PA
AGAP006327-PA	AGAP004093-PA
AGAP006348-PA	AGAP004094-PA
AGAP006353-PA	AGAP004108-PB
AGAP006400-PA	AGAP004110-PA
AGAP006430-PB	AGAP004146-PA

AGAP006459-PA	AGAP004148-PA
AGAP006469-PA	AGAP004161-PA
AGAP006478-PA	AGAP004164-PA
AGAP006485-PA	AGAP004192-PA
AGAP006487-PA	AGAP004212-PA
AGAP006497-PA	AGAP004235-PA
AGAP006502-PA	AGAP004237-PA
AGAP006548-PA	AGAP004247-PA
AGAP006607-PB	AGAP004247-PB
AGAP006614-PA	AGAP004286-PA
AGAP006615-PA	AGAP004318-PA
AGAP006660-PC	AGAP004321-PA
AGAP006670-PA	AGAP004324-PA
AGAP006674-PA	AGAP004349-PA
AGAP006676-PA	AGAP004362-PB
AGAP006684-PA	AGAP004378-PA
AGAP006733-PA	AGAP004395-PA
AGAP006766-PA	AGAP004407-PA
AGAP006782-PA	AGAP004418-PA
AGAP006804-PA	AGAP004422-PA
AGAP006828-PA	AGAP004441-PA
AGAP006830-PA	AGAP004443-PB
AGAP006847-PA	AGAP004455-PA
AGAP006848-PA	AGAP004462-PA
AGAP006859-PA	AGAP004481-PA
AGAP006863-PA	AGAP004484-PA
AGAP006871-PA	AGAP004514-PA
AGAP006884-PA	AGAP004520-PA
AGAP006895-PA	AGAP004533-PA
AGAP006918-PA	AGAP004551-PA
AGAP006935-PC	AGAP004556-PA

AGAP006936-PB
AGAP006937-PC
AGAP006946-PA
AGAP006996-PA
AGAP007024-PA
AGAP007033-PA
AGAP007036-PA
AGAP007059-PA
AGAP007060-PA
AGAP007082-PA
AGAP007088-PA
AGAP007103-PD
AGAP007120-PA
AGAP007121-PA
AGAP007125-PA
AGAP007157-PA
AGAP007162-PB
AGAP007202-PA
AGAP007203-PB
AGAP007208-PA
AGAP007218-PA
AGAP007247-PC
AGAP007249-PB
AGAP007250-PA
AGAP007252-PA
AGAP007263-PA
AGAP007297-PA
AGAP007304-PA
AGAP007339-PA
AGAP007345-PA
AGAP007380-PA
AGAP004566-PA
AGAP004606-PA
AGAP004609-PA
AGAP004611-PA
AGAP004618-PA
AGAP004625-PB
AGAP004631-PA
AGAP004638-PA
AGAP004641-PA
AGAP004645-PA
AGAP004653-PA
AGAP004674-PA
AGAP004677-PB
AGAP004689-PC
AGAP004719-PA
AGAP004723-PA
AGAP004739-PA
AGAP004743-PA
AGAP004744-PA
AGAP004758-PB
AGAP004773-PA
AGAP004794-PA
AGAP004802-PA
AGAP004807-PA
AGAP004809-PA
AGAP004811-PA
AGAP004832-PA
AGAP004845-PB
AGAP004849-PA
AGAP004855-PA
AGAP004864-PA

AGAP007393-PC	AGAP004867-PA
AGAP007400-PA	AGAP004868-PA
AGAP007406-PA	AGAP004877-PB
AGAP007411-PA	AGAP004880-PC
AGAP007460-PB	AGAP004886-PB
AGAP007473-PA	AGAP004887-PA
AGAP007475-PA	AGAP004895-PA
AGAP007505-PA	AGAP004919-PA
AGAP007507-PA	AGAP004940-PA
AGAP007529-PA	AGAP004960-PA
AGAP007543-PA	AGAP004975-PA
AGAP007565-PA	AGAP004990-PA
AGAP007574-PA	AGAP004991-PA
AGAP007575-PA	AGAP004996-PA
AGAP007580-PB	AGAP004997-PA
AGAP007613-PA	AGAP005003-PA
AGAP007625-PA	AGAP005015-PA
AGAP007643-PB	AGAP005032-PB
AGAP007643-PD	AGAP005046-PB
AGAP007644-PA	AGAP005061-PB
AGAP007665-PA	AGAP005076-PC
AGAP007666-PA	AGAP005081-PA
AGAP007668-PA	AGAP005095-PA
AGAP007684-PB	AGAP005108-PA
AGAP007699-PC	AGAP005109-PA
AGAP007705-PA	AGAP005118-PA
AGAP007722-PA	AGAP005122-PA
AGAP007724-PC	AGAP005129-PA
AGAP007790-PD	AGAP005131-PA
AGAP007791-PA	AGAP005134-PA
AGAP007791-PE	AGAP005160-PE

AGAP007806-PA
AGAP007827-PA
AGAP007864-PC
AGAP007867-PA
AGAP007942-PB
AGAP007963-PA
AGAP008006-PA
AGAP008051-PA
AGAP008052-PA
AGAP008054-PD
AGAP008061-PA
AGAP008073-PA
AGAP008096-PA
AGAP008113-PB
AGAP008138-PA
AGAP008140-PA
AGAP008176-PA
AGAP008225-PA
AGAP008241-PA
AGAP008265-PA
AGAP008327-PA
AGAP008329-PC
AGAP008339-PA
AGAP008345-PA
AGAP008370-PA
AGAP008374-PA
AGAP008398-PA
AGAP008403-PA
AGAP008405-PB
AGAP008408-PF
AGAP008425-PA
AGAP005163-PA
AGAP005197-PA
AGAP005234-PB
AGAP005265-PA
AGAP005306-PA
AGAP005316-PA
AGAP005327-PA
AGAP005335-PA
AGAP005350-PA
AGAP005365-PA
AGAP005376-PA
AGAP005381-PC
AGAP005386-PA
AGAP005402-PA
AGAP005410-PC
AGAP005423-PA
AGAP005427-PA
AGAP005451-PA
AGAP005459-PA
AGAP005462-PA
AGAP005486-PA
AGAP005540-PA
AGAP005558-PA
AGAP005559-PA
AGAP005569-PA
AGAP005581-PA
AGAP005597-PA
AGAP005603-PA
AGAP005609-PB
AGAP005613-PB
AGAP005615-PA

AGAP008432-PD	AGAP005618-PA
AGAP008440-PA	AGAP005627-PD
AGAP008465-PA	AGAP005641-PA
AGAP008495-PA	AGAP005642-PA
AGAP008499-PA	AGAP005644-PA
AGAP008503-PB	AGAP005662-PA
AGAP008548-PA	AGAP005663-PA
AGAP008582-PA	AGAP005670-PA
AGAP008584-PA	AGAP005671-PA
AGAP008615-PA	AGAP005674-PA
AGAP008634-PA	AGAP005685-PA
AGAP008653-PA	AGAP005686-PA
AGAP008667-PB	AGAP005690-PA
AGAP008719-PA	AGAP005693-PA
AGAP008724-PA	AGAP005695-PA
AGAP008727-PF	AGAP005696-PA
AGAP008783-PA	AGAP005723-PA
AGAP008802-PA	AGAP005739-PA
AGAP008816-PA	AGAP005744-PA
AGAP008817-PA	AGAP005749-PA
AGAP008822-PA	AGAP005766-PA
AGAP008835-PA	AGAP005802-PA
AGAP008838-PA	AGAP005813-PA
AGAP008867-PA	AGAP005838-PA
AGAP008878-PA	AGAP005845-PA
AGAP008883-PA	AGAP005858-PA
AGAP008893-PA	AGAP005861-PB
AGAP008895-PA	AGAP005914-PA
AGAP008898-PA	AGAP005947-PA
AGAP008909-PA	AGAP005960-PA
AGAP008952-PA	AGAP005979-PA

AGAP008955-PA
AGAP008988-PA
AGAP009031-PA
AGAP009035-PA
AGAP009072-PA
AGAP009119-PA
AGAP009121-PA
AGAP009122-PA
AGAP009142-PA
AGAP009145-PA
AGAP009146-PA
AGAP009154-PA
AGAP009173-PA
AGAP009179-PA
AGAP009184-PA
AGAP009193-PA
AGAP009194-PA
AGAP009214-PA
AGAP009216-PA
AGAP009234-PA
AGAP009264-PB
AGAP009271-PA
AGAP009305-PA
AGAP009310-PA
AGAP009317-PA
AGAP009324-PA
AGAP009330-PA
AGAP009336-PA
AGAP009347-PB
AGAP009348-PA
AGAP009379-PA
AGAP005981-PA
AGAP005991-PA
AGAP005996-PA
AGAP005997-PA
AGAP005998-PA
AGAP006000-PA
AGAP006009-PA
AGAP006010-PA
AGAP006011-PA
AGAP006012-PA
AGAP006037-PA
AGAP006070-PA
AGAP006090-PA
AGAP006095-PA
AGAP006099-PA
AGAP006102-PA
AGAP006103-PA
AGAP006108-PA
AGAP006117-PA
AGAP006141-PB
AGAP006145-PA
AGAP006146-PA
AGAP006160-PA
AGAP006161-PA
AGAP006179-PB
AGAP006186-PA
AGAP006208-PA
AGAP006209-PA
AGAP006260-PD
AGAP006275-PA
AGAP006278-PA

AGAP009380-PA
AGAP009405-PA
AGAP009431-PB
AGAP009441-PB
AGAP009447-PJ
AGAP009507-PA
AGAP009508-PA
AGAP009510-PA
AGAP009537-PA
AGAP009584-PA
AGAP009587-PA
AGAP009595-PA
AGAP009621-PA
AGAP009623-PA
AGAP009633-PA
AGAP009644-PA
AGAP009652-PA
AGAP009660-PA
AGAP009667-PA
AGAP009670-PA
AGAP009670-PB
AGAP009672-PA
AGAP009685-PA
AGAP009691-PA
AGAP009692-PA
AGAP009738-PA
AGAP009751-PA
AGAP009752-PA
AGAP009753-PA
AGAP009754-PA
AGAP009755-PA
AGAP006283-PB
AGAP006327-PA
AGAP006342-PA
AGAP006343-PA
AGAP006348-PA
AGAP006353-PA
AGAP006365-PA
AGAP006398-PA
AGAP006400-PA
AGAP006424-PA
AGAP006430-PB
AGAP006456-PA
AGAP006459-PA
AGAP006468-PA
AGAP006469-PA
AGAP006478-PA
AGAP006485-PA
AGAP006487-PA
AGAP006489-PA
AGAP006497-PA
AGAP006502-PA
AGAP006518-PA
AGAP006548-PA
AGAP006607-PB
AGAP006613-PB
AGAP006614-PA
AGAP006615-PA
AGAP006660-PC
AGAP006668-PA
AGAP006670-PA
AGAP006674-PA

AGAP009783-PA
AGAP009788-PA
AGAP009790-PA
AGAP009823-PA
AGAP009833-PA
AGAP009842-PA
AGAP009844-PA
AGAP009859-PA
AGAP009861-PA
AGAP009863-PA
AGAP009865-PA
AGAP009873-PA
AGAP009875-PA
AGAP009901-PA
AGAP009920-PA
AGAP009968-PA
AGAP009998-PA
AGAP010007-PA
AGAP010025-PA
AGAP010047-PA
AGAP010056-PA
AGAP010065-PA
AGAP010095-PA
AGAP010097-PA
AGAP010100-PA
AGAP010121-PA
AGAP010123-PA
AGAP010133-PA
AGAP010142-PB
AGAP010145-PA
AGAP010147-PE
AGAP006675-PA
AGAP006676-PA
AGAP006684-PA
AGAP006733-PA
AGAP006766-PA
AGAP006782-PA
AGAP006804-PA
AGAP006808-PA
AGAP006828-PA
AGAP006829-PA
AGAP006830-PA
AGAP006847-PA
AGAP006848-PA
AGAP006859-PA
AGAP006863-PA
AGAP006871-PA
AGAP006884-PA
AGAP006895-PA
AGAP006911-PA
AGAP006918-PA
AGAP006935-PC
AGAP006936-PB
AGAP006937-PC
AGAP006946-PA
AGAP006958-PA
AGAP006962-PA
AGAP006996-PA
AGAP007024-PA
AGAP007033-PA
AGAP007036-PA
AGAP007039-PA

AGAP010159-PA	AGAP007059-PA
AGAP010173-PA	AGAP007060-PA
AGAP010174-PA	AGAP007082-PA
AGAP010175-PD	AGAP007088-PA
AGAP010177-PA	AGAP007103-PD
AGAP010181-PA	AGAP007112-PA
AGAP010188-PA	AGAP007120-PA
AGAP010191-PA	AGAP007121-PA
AGAP010193-PA	AGAP007125-PA
AGAP010212-PA	AGAP007157-PA
AGAP010216-PA	AGAP007162-PB
AGAP010220-PA	AGAP007172-PB
AGAP010240-PA	AGAP007202-PA
AGAP010252-PA	AGAP007203-PB
AGAP010253-PA	AGAP007208-PA
AGAP010257-PA	AGAP007218-PA
AGAP010268-PA	AGAP007247-PC
AGAP010278-PA	AGAP007249-PB
AGAP010280-PA	AGAP007250-PA
AGAP010298-PA	AGAP007252-PA
AGAP010307-PA	AGAP007263-PA
AGAP010337-PD	AGAP007297-PA
AGAP010338-PA	AGAP007304-PA
AGAP010347-PA	AGAP007309-PA
AGAP010362-PA	AGAP007325-PB
AGAP010375-PA	AGAP007339-PA
AGAP010392-PA	AGAP007345-PA
AGAP010404-PB	AGAP007362-PB
AGAP010421-PA	AGAP007380-PA
AGAP010477-PB	AGAP007393-PC
AGAP010479-PA	AGAP007400-PA

AGAP010496-PB	AGAP007406-PA
AGAP010514-PA	AGAP007407-PA
AGAP010517-PB	AGAP007411-PA
AGAP010557-PA	AGAP007460-PB
AGAP010564-PA	AGAP007473-PA
AGAP010586-PA	AGAP007475-PA
AGAP010591-PA	AGAP007505-PA
AGAP010592-PA	AGAP007507-PA
AGAP010608-PA	AGAP007529-PA
AGAP010613-PA	AGAP007541-PA
AGAP010640-PA	AGAP007543-PA
AGAP010657-PA	AGAP007565-PA
AGAP010661-PA	AGAP007574-PA
AGAP010675-PA	AGAP007575-PA
AGAP010682-PA	AGAP007580-PB
AGAP010718-PA	AGAP007613-PA
AGAP010730-PA	AGAP007625-PA
AGAP010731-PA	AGAP007643-PB
AGAP010733-PA	AGAP007643-PD
AGAP010755-PA	AGAP007644-PA
AGAP010764-PA	AGAP007663-PA
AGAP010815-PA	AGAP007665-PA
AGAP010822-PA	AGAP007666-PA
AGAP010823-PA	AGAP007668-PA
AGAP010848-PA	AGAP007684-PB
AGAP010876-PA	AGAP007699-PC
AGAP010900-PA	AGAP007705-PA
AGAP010902-PA	AGAP007722-PA
AGAP010929-PA	AGAP007724-PC
AGAP011039-PA	AGAP007740-PA
AGAP011048-PA	AGAP007777-PA

AGAP011050-PA	AGAP007780-PB
AGAP011051-PA	AGAP007790-PD
AGAP011054-PA	AGAP007791-PA
AGAP011062-PA	AGAP007791-PE
AGAP011076-PA	AGAP007806-PA
AGAP011077-PA	AGAP007827-PA
AGAP011084-PA	AGAP007841-PA
AGAP011092-PA	AGAP007864-PC
AGAP011098-PA	AGAP007867-PA
AGAP011105-PA	AGAP007921-PA
AGAP011107-PA	AGAP007942-PB
AGAP011115-PA	AGAP007963-PA
AGAP011119-PA	AGAP007966-PC
AGAP011130-PA	AGAP008006-PA
AGAP011131-PA	AGAP008013-PA
AGAP011140-PA	AGAP008043-PA
AGAP011150-PA	AGAP008051-PA
AGAP011157-PA	AGAP008052-PA
AGAP011159-PA	AGAP008054-PD
AGAP011165-PA	AGAP008060-PA
AGAP011166-PA	AGAP008061-PA
AGAP011173-PA	AGAP008073-PA
AGAP011183-PA	AGAP008075-PC
AGAP011190-PA	AGAP008096-PA
AGAP011193-PA	AGAP008113-PB
AGAP011197-PA	AGAP008138-PA
AGAP011203-PA	AGAP008140-PA
AGAP011225-PA	AGAP008176-PA
AGAP011226-PA	AGAP008225-PA
AGAP011239-PA	AGAP008241-PA
AGAP011276-PA	AGAP008265-PA

AGAP011277-PA	AGAP008327-PA
AGAP011284-PA	AGAP008329-PC
AGAP011298-PA	AGAP008332-PA
AGAP011302-PA	AGAP008339-PA
AGAP011309-PA	AGAP008345-PA
AGAP011319-PA	AGAP008364-PA
AGAP011325-PA	AGAP008370-PA
AGAP011329-PA	AGAP008374-PA
AGAP011334-PA	AGAP008398-PA
AGAP011346-PA	AGAP008403-PA
AGAP011354-PA	AGAP008405-PB
AGAP011367-PA	AGAP008408-PF
AGAP011383-PA	AGAP008425-PA
AGAP011423-PA	AGAP008432-PD
AGAP011424-PA	AGAP008440-PA
AGAP011438-PA	AGAP008465-PA
AGAP011442-PA	AGAP008495-PA
AGAP011446-PA	AGAP008499-PA
AGAP011453-PA	AGAP008503-PB
AGAP011457-PA	AGAP008548-PA
AGAP011476-PA	AGAP008582-PA
AGAP011477-PA	AGAP008584-PA
AGAP011478-PA	AGAP008615-PA
AGAP011503-PA	AGAP008634-PA
AGAP011504-PA	AGAP008653-PA
AGAP011505-PA	AGAP008667-PB
AGAP011514-PA	AGAP008719-PA
AGAP011521-PA	AGAP008724-PA
AGAP011532-PA	AGAP008727-PF
AGAP011581-PA	AGAP008774-PA
AGAP011590-PA	AGAP008783-PA

AGAP011604-PA	AGAP008802-PA
AGAP011608-PA	AGAP008816-PA
AGAP011634-PA	AGAP008817-PA
AGAP011635-PA	AGAP008822-PA
AGAP011682-PA	AGAP008835-PA
AGAP011683-PA	AGAP008837-PA
AGAP011685-PA	AGAP008838-PA
AGAP011687-PA	AGAP008867-PA
AGAP011700-PA	AGAP008878-PA
AGAP011706-PA	AGAP008883-PA
AGAP011765-PA	AGAP008885-PA
AGAP011768-PA	AGAP008893-PA
AGAP011777-PA	AGAP008895-PA
AGAP011780-PA	AGAP008898-PA
AGAP011781-PA	AGAP008909-PA
AGAP011783-PA	AGAP008916-PA
AGAP011788-PA	AGAP008952-PA
AGAP011789-PA	AGAP008955-PA
AGAP011790-PB	AGAP008963-PA
AGAP011791-PA	AGAP008988-PA
AGAP011792-PA	AGAP009031-PA
AGAP011802-PA	AGAP009035-PA
AGAP011824-PA	AGAP009072-PA
AGAP011828-PA	AGAP009119-PA
AGAP011829-PA	AGAP009121-PA
AGAP011832-PA	AGAP009122-PA
AGAP011833-PA	AGAP009142-PA
AGAP011842-PA	AGAP009145-PA
AGAP011884-PA	AGAP009146-PA
AGAP011896-PA	AGAP009154-PA
AGAP011919-PA	AGAP009173-PA

AGAP011920-PA	AGAP009179-PA
AGAP011931-PA	AGAP009184-PA
AGAP011936-PA	AGAP009193-PA
AGAP011937-PA	AGAP009194-PA
AGAP012008-PA	AGAP009214-PA
AGAP012041-PA	AGAP009216-PA
AGAP012048-PA	AGAP009234-PA
AGAP012056-PA	AGAP009264-PB
AGAP012071-PA	AGAP009271-PA
AGAP012081-PA	AGAP009305-PA
AGAP012100-PA	AGAP009310-PA
AGAP012108-PA	AGAP009317-PA
AGAP012126-PA	AGAP009324-PA
AGAP012135-PA	AGAP009330-PA
AGAP012136-PA	AGAP009336-PA
AGAP012138-PA	AGAP009347-PB
AGAP012144-PA	AGAP009348-PA
AGAP012165-PA	AGAP009379-PA
AGAP012168-PA	AGAP009380-PA
AGAP012184-PA	AGAP009388-PB
AGAP012188-PA	AGAP009405-PA
AGAP012204-PA	AGAP009431-PB
AGAP012284-PA	AGAP009441-PB
AGAP012304-PA	AGAP009447-PJ
AGAP012305-PA	AGAP009507-PA
AGAP012317-PA	AGAP009508-PA
AGAP012325-PA	AGAP009510-PA
AGAP012334-PA	AGAP009537-PA
AGAP012339-PA	AGAP009584-PA
AGAP012352-PA	AGAP009587-PA
AGAP012356-PA	AGAP009595-PA

AGAP012364-PA	AGAP009604-PA
AGAP012374-PA	AGAP009621-PA
AGAP012380-PA	AGAP009623-PA
AGAP012395-PA	AGAP009633-PA
AGAP012407-PA	AGAP009644-PA
AGAP012425-PA	AGAP009652-PA
AGAP012504-PA	AGAP009660-PA
AGAP012515-PA	AGAP009667-PA
AGAP012529-PA	AGAP009670-PA
AGAP012560-PA	AGAP009670-PB
AGAP012577-PA	AGAP009672-PA
AGAP012608-PA	AGAP009685-PA
AGAP012614-PA	AGAP009691-PA
AGAP012650-PA	AGAP009692-PA
AGAP012656-PA	AGAP009738-PA
AGAP012666-PA	AGAP009751-PA
AGAP012703-PA	AGAP009752-PA
AGAP012750-PA	AGAP009753-PA
AGAP012823-PA	AGAP009754-PA
AGAP012837-PA	AGAP009755-PA
AGAP012895-PA	AGAP009783-PA
AGAP012990-PA	AGAP009788-PA
AGAP013036-PA	AGAP009790-PA
AGAP013066-PA	AGAP009823-PA
AGAP013078-PA	AGAP009833-PA
AGAP013090-PA	AGAP009842-PA
AGAP013092-PA	AGAP009844-PA
AGAP013105-PA	AGAP009859-PA
AGAP013106-PA	AGAP009861-PA
AGAP013112-PA	AGAP009863-PA
AGAP013114-PA	AGAP009865-PA

AGAP013153-PA
AGAP013164-PA
AGAP013185-PA
AGAP013221-PA
AGAP013228-PA
AGAP013229-PA
AGAP013231-PA
AGAP013272-PA
AGAP013286-PC
AGAP013296-PA
AGAP013308-PA
AGAP013313-PA
AGAP013318-PA
AGAP013319-PA
AGAP013336-PA
AGAP013347-PA
AGAP013365-PA
AGAP013375-PA
AGAP013376-PA
AGAP013398-PA
AGAP013400-PA
AGAP013442-PB
AGAP013451-PA
AGAP013471-PA
AGAP013481-PB
AGAP013481-PC
AGAP013481-PD
AGAP013481-PE
AGAP013742-PA
AGAP013769-PA
AGAP028011-PA
AGAP009873-PA
AGAP009875-PA
AGAP009901-PA
AGAP009920-PA
AGAP009968-PA
AGAP009998-PA
AGAP010007-PA
AGAP010025-PA
AGAP010047-PA
AGAP010052-PA
AGAP010056-PA
AGAP010065-PA
AGAP010095-PA
AGAP010097-PA
AGAP010100-PA
AGAP010121-PA
AGAP010123-PA
AGAP010130-PB
AGAP010133-PA
AGAP010142-PB
AGAP010145-PA
AGAP010147-PE
AGAP010159-PA
AGAP010173-PA
AGAP010174-PA
AGAP010175-PD
AGAP010177-PA
AGAP010181-PA
AGAP010188-PA
AGAP010191-PA
AGAP010193-PA

AGAP028042-PA	AGAP010212-PA
AGAP028064-PA	AGAP010216-PA
AGAP028068-PA	AGAP010220-PA
AGAP028143-PA	AGAP010240-PA
AGAP028149-PA	AGAP010252-PA
AGAP028178-PA	AGAP010253-PA
AGAP028194-PA	AGAP010257-PA
AGAP028196-PA	AGAP010268-PA
AGAP028200-PA	AGAP010278-PA
AGAP028204-PA	AGAP010280-PA
AGAP028208-PA	AGAP010298-PA
AGAP028366-PA	AGAP010307-PA
AGAP028400-PA	AGAP010337-PD
AGAP028413-PA	AGAP010338-PA
AGAP000862-PA	AGAP010347-PA
AGAP001325-PA	AGAP010362-PA
AGAP001438-PB	AGAP010375-PA
AGAP001545-PA	AGAP010392-PA
AGAP001657-PA	AGAP010404-PB
AGAP001675-PA	AGAP010421-PA
AGAP001721-PA	AGAP010477-PB
AGAP001760-PA	AGAP010479-PA
AGAP001797-PJ	AGAP010496-PB
AGAP001805-PA	AGAP010514-PA
AGAP001823-PA	AGAP010517-PB
AGAP001825-PA	AGAP010557-PA
AGAP001827-PA	AGAP010564-PA
AGAP001902-PA	AGAP010586-PA
AGAP001995-PA	AGAP010590-PA
AGAP002049-PA	AGAP010591-PA
AGAP002055-PA	AGAP010592-PA

AGAP002170-PA
AGAP002198-PA
AGAP002350-PA
AGAP002491-PA
AGAP002499-PA
AGAP002919-PA
AGAP003592-PB
AGAP003598-PA
AGAP003807-PA
AGAP003935-PA
AGAP004481-PA
AGAP004758-PB
AGAP004811-PA
AGAP004868-PA
AGAP005122-PA
AGAP005131-PA
AGAP005160-PE
AGAP005265-PA
AGAP005365-PA
AGAP005690-PA
AGAP006099-PA
AGAP006161-PA
AGAP006260-PD
AGAP006343-PA
AGAP006456-PA
AGAP006468-PA
AGAP006518-PA
AGAP006668-PA
AGAP006675-PA
AGAP006808-PA
AGAP006911-PA
AGAP010608-PA
AGAP010613-PA
AGAP010640-PA
AGAP010657-PA
AGAP010661-PA
AGAP010675-PA
AGAP010682-PA
AGAP010718-PA
AGAP010730-PA
AGAP010731-PA
AGAP010733-PA
AGAP010755-PA
AGAP010764-PA
AGAP010815-PA
AGAP010822-PA
AGAP010823-PA
AGAP010848-PA
AGAP010876-PA
AGAP010900-PA
AGAP010902-PA
AGAP010929-PA
AGAP010933-PA
AGAP010957-PA
AGAP011039-PA
AGAP011048-PA
AGAP011050-PA
AGAP011051-PA
AGAP011054-PA
AGAP011062-PA
AGAP011076-PA
AGAP011077-PA

AGAP006962-PA
AGAP007362-PB
AGAP007407-PA
AGAP007841-PA
AGAP007921-PA
AGAP007966-PC
AGAP008364-PA
AGAP008837-PA
AGAP008885-PA
AGAP008963-PA
AGAP009388-PB
AGAP010130-PB
AGAP010590-PA
AGAP010933-PA
AGAP000767-PA
AGAP001056-PA
AGAP001082-PA
AGAP001127-PA
AGAP001219-PA
AGAP001274-PA
AGAP001321-PA
AGAP001613-PA
AGAP001621-PB
AGAP001711-PA
AGAP001746-PA
AGAP001799-PB
AGAP001957-PB
AGAP001989-PA
AGAP002078-PA
AGAP002122-PA
AGAP002171-PA
AGAP011084-PA
AGAP011092-PA
AGAP011098-PA
AGAP011105-PA
AGAP011107-PA
AGAP011115-PA
AGAP011119-PA
AGAP011130-PA
AGAP011131-PA
AGAP011140-PA
AGAP011150-PA
AGAP011157-PA
AGAP011159-PA
AGAP011165-PA
AGAP011166-PA
AGAP011173-PA
AGAP011183-PA
AGAP011190-PA
AGAP011193-PA
AGAP011197-PA
AGAP011203-PA
AGAP011225-PA
AGAP011226-PA
AGAP011239-PA
AGAP011276-PA
AGAP011277-PA
AGAP011284-PA
AGAP011298-PA
AGAP011302-PA
AGAP011309-PA
AGAP011319-PA

AGAP002218-PA
AGAP002219-PA
AGAP002468-PA
AGAP003069-PA
AGAP003136-PA
AGAP003178-PA
AGAP003289-PA
AGAP003308-PA
AGAP003541-PC
AGAP003556-PA
AGAP003878-PA
AGAP003971-PA
AGAP004148-PA
AGAP004247-PA
AGAP004247-PB
AGAP004739-PA
AGAP004864-PA
AGAP004990-PA
AGAP005306-PA
AGAP005350-PA
AGAP005386-PA
AGAP005410-PC
AGAP005597-PA
AGAP005613-PB
AGAP005670-PA
AGAP005802-PA
AGAP006141-PB
AGAP006342-PA
AGAP006489-PA
AGAP006829-PA
AGAP006958-PA
AGAP011325-PA
AGAP011329-PA
AGAP011334-PA
AGAP011346-PA
AGAP011354-PA
AGAP011367-PA
AGAP011369-PA
AGAP011383-PA
AGAP011423-PA
AGAP011424-PA
AGAP011438-PA
AGAP011442-PA
AGAP011446-PA
AGAP011453-PA
AGAP011457-PA
AGAP011476-PA
AGAP011477-PA
AGAP011478-PA
AGAP011503-PA
AGAP011504-PA
AGAP011505-PA
AGAP011514-PA
AGAP011521-PA
AGAP011532-PA
AGAP011581-PA
AGAP011590-PA
AGAP011604-PA
AGAP011608-PA
AGAP011634-PA
AGAP011635-PA
AGAP011682-PA

AGAP007039-PA	AGAP011683-PA
AGAP007172-PB	AGAP011685-PA
AGAP007325-PB	AGAP011687-PA
AGAP008332-PA	AGAP011700-PA
AGAP008916-PA	AGAP011706-PA
AGAP010052-PA	AGAP011765-PA
NA	AGAP011768-PA
NA	AGAP011777-PA
NA	AGAP011780-PA
NA	AGAP011781-PA
NA	AGAP011783-PA
NA	AGAP011788-PA
NA	AGAP011789-PA
NA	AGAP011790-PB
NA	AGAP011791-PA
NA	AGAP011792-PA
NA	AGAP011802-PA
NA	AGAP011824-PA
NA	AGAP011828-PA
NA	AGAP011829-PA
NA	AGAP011832-PA
NA	AGAP011833-PA
NA	AGAP011842-PA
NA	AGAP011884-PA
NA	AGAP011896-PA
NA	AGAP011919-PA
NA	AGAP011920-PA
NA	AGAP011931-PA
NA	AGAP011936-PA
NA	AGAP011937-PA
NA	AGAP012008-PA

NA	AGAP012041-PA
NA	AGAP012048-PA
NA	AGAP012056-PA
NA	AGAP012071-PA
NA	AGAP012081-PA
NA	AGAP012100-PA
NA	AGAP012108-PA
NA	AGAP012126-PA
NA	AGAP012135-PA
NA	AGAP012136-PA
NA	AGAP012138-PA
NA	AGAP012144-PA
NA	AGAP012165-PA
NA	AGAP012168-PA
NA	AGAP012184-PA
NA	AGAP012188-PA
NA	AGAP012204-PA
NA	AGAP012284-PA
NA	AGAP012304-PA
NA	AGAP012305-PA
NA	AGAP012317-PA
NA	AGAP012325-PA
NA	AGAP012334-PA
NA	AGAP012339-PA
NA	AGAP012352-PA
NA	AGAP012356-PA
NA	AGAP012364-PA
NA	AGAP012374-PA
NA	AGAP012380-PA
NA	AGAP012395-PA
NA	AGAP012407-PA

NA	AGAP012425-PA
NA	AGAP012504-PA
NA	AGAP012515-PA
NA	AGAP012529-PA
NA	AGAP012560-PA
NA	AGAP012577-PA
NA	AGAP012608-PA
NA	AGAP012614-PA
NA	AGAP012650-PA
NA	AGAP012656-PA
NA	AGAP012666-PA
NA	AGAP012703-PA
NA	AGAP012750-PA
NA	AGAP012823-PA
NA	AGAP012837-PA
NA	AGAP012895-PA
NA	AGAP012990-PA
NA	AGAP013036-PA
NA	AGAP013066-PA
NA	AGAP013078-PA
NA	AGAP013090-PA
NA	AGAP013092-PA
NA	AGAP013105-PA
NA	AGAP013106-PA
NA	AGAP013112-PA
NA	AGAP013114-PA
NA	AGAP013153-PA
NA	AGAP013164-PA
NA	AGAP013185-PA
NA	AGAP013221-PA
NA	AGAP013228-PA

NA	AGAP013229-PA
NA	AGAP013231-PA
NA	AGAP013272-PA
NA	AGAP013286-PC
NA	AGAP013296-PA
NA	AGAP013308-PA
NA	AGAP013313-PA
NA	AGAP013318-PA
NA	AGAP013319-PA
NA	AGAP013336-PA
NA	AGAP013347-PA
NA	AGAP013365-PA
NA	AGAP013375-PA
NA	AGAP013376-PA
NA	AGAP013398-PA
NA	AGAP013400-PA
NA	AGAP013442-PB
NA	AGAP013451-PA
NA	AGAP013471-PA
NA	AGAP013481-PB
NA	AGAP013481-PC
NA	AGAP013481-PD
NA	AGAP013481-PE
NA	AGAP013742-PA
NA	AGAP013769-PA
NA	AGAP028011-PA
NA	AGAP028042-PA
NA	AGAP028064-PA
NA	AGAP028068-PA
NA	AGAP028143-PA
NA	AGAP028149-PA

NA	AGAP028178-PA
NA	AGAP028194-PA
NA	AGAP028196-PA
NA	AGAP028200-PA
NA	AGAP028204-PA
NA	AGAP028208-PA
NA	AGAP028366-PA
NA	AGAP028400-PA
NA	AGAP028413-PA
NA	AGAP000005-PA
NA	AGAP000044-PA
NA	AGAP000167-PA
NA	AGAP000180-PA
NA	AGAP000260-PC
NA	AGAP000261-PA
NA	AGAP000270-PA
NA	AGAP000278-PA
NA	AGAP000291-PA
NA	AGAP000297-PA
NA	AGAP000305-PA
NA	AGAP000306-PA
NA	AGAP000352-PA
NA	AGAP000359-PA
NA	AGAP000403-PA
NA	AGAP000415-PA
NA	AGAP000416-PC
NA	AGAP000462-PB
NA	AGAP000526-PA
NA	AGAP000536-PA
NA	AGAP000538-PA
NA	AGAP000541-PB

NA	AGAP000572-PA
NA	AGAP000615-PA
NA	AGAP000622-PB
NA	AGAP000625-PA
NA	AGAP000654-PA
NA	AGAP000739-PA
NA	AGAP000749-PA
NA	AGAP000755-PA
NA	AGAP000820-PA
NA	AGAP000883-PA
NA	AGAP000927-PB
NA	AGAP000949-PA
NA	AGAP000950-PA
NA	AGAP000952-PA
NA	AGAP001023-PF
NA	AGAP001053-PG
NA	AGAP001127-PA
NA	AGAP001138-PA
NA	AGAP001177-PA
NA	AGAP001194-PA
NA	AGAP001245-PA
NA	AGAP001274-PA
NA	AGAP001313-PA
NA	AGAP001346-PA
NA	AGAP001375-PA
NA	AGAP001380-PA
NA	AGAP001408-PA
NA	AGAP001424-PA
NA	AGAP001438-PB
NA	AGAP001545-PA
NA	AGAP001613-PA

NA	AGAP001630-PA
NA	AGAP001645-PA
NA	AGAP001657-PA
NA	AGAP001668-PA
NA	AGAP001717-PA
NA	AGAP001721-PA
NA	AGAP001768-PB
NA	AGAP001799-PA
NA	AGAP001810-PA
NA	AGAP001825-PA
NA	AGAP001826-PA
NA	AGAP001827-PA
NA	AGAP001903-PA
NA	AGAP001910-PA
NA	AGAP001914-PA
NA	AGAP001919-PA
NA	AGAP001928-PA
NA	AGAP001956-PA
NA	AGAP002020-PA
NA	AGAP002063-PA
NA	AGAP002076-PA
NA	AGAP002078-PA
NA	AGAP002171-PA
NA	AGAP002198-PA
NA	AGAP002218-PA
NA	AGAP002251-PA
NA	AGAP002341-PA
NA	AGAP002346-PA
NA	AGAP002350-PA
NA	AGAP002350-PB
NA	AGAP000315-PA

NA	AGAP000437-PA
NA	AGAP000508-PA
NA	AGAP000651-PC
NA	AGAP000672-PB
NA	AGAP000720-PA
NA	AGAP000756-PB
NA	AGAP000897-PA
NA	AGAP000941-PA
NA	AGAP000970-PA
NA	AGAP000988-PA
NA	AGAP000989-PA
NA	AGAP000994-PA
NA	AGAP001007-PA
NA	AGAP001053-PB
NA	AGAP001053-PE
NA	AGAP001064-PA
NA	AGAP001151-PA
NA	AGAP001246-PA
NA	AGAP001249-PA
NA	AGAP001345-PA
NA	AGAP001356-PA
NA	AGAP001372-PA
NA	AGAP001377-PA
NA	AGAP001420-PA
NA	AGAP001459-PC
NA	AGAP001502-PA
NA	AGAP001617-PB
NA	AGAP001621-PB
NA	AGAP001622-PA
NA	AGAP001624-PA
NA	AGAP001659-PA

NA	AGAP001701-PA
NA	AGAP001718-PA
NA	AGAP001749-PA
NA	AGAP001760-PA
NA	AGAP001791-PA
NA	AGAP001797-PD
NA	AGAP001797-PI
NA	AGAP001911-PA
NA	AGAP001930-PB
NA	AGAP001973-PA
NA	AGAP001995-PA
NA	AGAP002005-PA
NA	AGAP002049-PA
NA	AGAP002085-PC
NA	AGAP002122-PA
NA	AGAP002227-PA
NA	AGAP002298-PA
NA	AGAP002306-PA

Appendix B. Proteins down regulated in cadmium treated mosquitoes

Protein stable ID	Gene name	InterPro short description
AGAP001151-PA		14-3-3
AGAP003212-PA	Adrenodoxin	2Fe-2S_ferredoxin-type
AGAP007309-PA		2Fe-2S_ferredoxin-type
AGAP003095-PA		6-blade_b-propeller_TolB-like
AGAP004065-PA		6-blade_b-propeller_TolB-like
AGAP004324-PA		6-blade_b-propeller_TolB-like
AGAP001911-PA	RpL6	60S_ribosomal_L6E
AGAP003216-PA		AAA+_ATPase
AGAP005662-PA		Acyl-CoA_DH_CS
AGAP003968-PA		Adenylat/UMP-CMP_kin
AGAP003581-PA		ADH_SF_Zn-type
AGAP003578-PA		Aldehyde_DH_dom
AGAP000941-PA		Alpha-crystallin/HSP
AGAP003790-PC	ANXB9	Annexin
AGAP003462-PA		Arg_MeTrfase
AGAP003277-PD		Aspartic_peptidase
AGAP004641-PA		ATP11
AGAP001138-PA		ATPase_B_chain/sub_B/MI25
AGAP005845-PA		ATPase_V1-cplx_csu
AGAP002473-PA	VATF	ATPase_V1-cplx_fsu_euk
AGAP001624-PA		ATPase_V1-cplx_s1su
AGAP003879-PA		ATPase_V1-cplx_s1su
AGAP002401-PA		ATPase_V1/A1-cplx_esu
AGAP001194-PA		Beta-lactamas-like
AGAP003337-PA		Beta-lactamas-like
AGAP007112-PA		BRCT_dom
AGAP003048-PA		Brix
AGAP004556-PA		Brix
AGAP003625-PA	CTL8	C-type_lectin

AGAP005335-PA	CTL4	C-type_lectin
AGAP000749-PA		Calponin_repeat
AGAP001313-PA		Calponin_repeat
AGAP004895-PA		Carbonic_anhydrase_a
AGAP001914-PA		Cdc37
AGAP003612-PA		CDV3
AGAP000897-PA		CHCH
AGAP000988-PA	CPAP3-A1c	Chitin-bd_dom
AGAP000989-PA	CPAP3-A1a	Chitin-bd_dom
AGAP001356-PA	ACE1	Cholinesterase
AGAP003008-PB		ClpA/B
AGAP004940-PA		cNMP-bd_dom
AGAP002559-PB		CRAL-bd_toc_tran
AGAP002661-PA		Crotonase_core_superfam
AGAP006117-PA		CS_dom
AGAP006108-PA		CSP_DNA-bd
AGAP004773-PA		DH_E1
AGAP002521-PB		DH_sc/Rdtase_SDR
AGAP002534-PB		DH_sc/Rdtase_SDR
AGAP003414-PB		DH_sc/Rdtase_SDR
AGAP001919-PA		Disulphide_isomerase
AGAP006398-PA		DM9_repeat
AGAP009604-PA		DM9_repeat
AGAP000970-PA		DnaJ_domain
AGAP001810-PA		DnaJ_domain
AGAP004849-PA		DnaJ_domain
AGAP007663-PA		DUF1397
AGAP001346-PA		DUF753
AGAP003919-PA		DUF866_euk
AGAP003360-PA		Dynein_light-rel
AGAP000927-PB		EF_hand_dom

AGAP001177-PA		EF_hand_dom
AGAP001622-PA		EF_hand_dom
AGAP004645-PA		EF_hand_dom
AGAP006179-PB		EF_hand_dom
AGAP010957-PA		EF_hand_dom
AGAP004631-PA		EF-hand-dom_pair
AGAP002739-PA		EGF-type_Asp/Asn_hydroxyl_site
AGAP006613-PB	eIF3j	eIF3j
AGAP001380-PA		eIF6
AGAP002925-PC		Endoribonuclease_XendoU
AGAP004653-PA		ET-Flavoprotein_bsu_CS
AGAP002564-PE		FBA_I
AGAP002464-PA		Ferritin
AGAP002465-PA		Ferritin
AGAP002005-PA		Fibrinogen_a/b/g_C_dom
AGAP004997-PA		Fibrinogen_a/b/g_C_dom
AGAP004807-PA	GALE7	Galectin_CRD
AGAP002355-PB		Gln_synth_cat_dom
AGAP002500-PA		Glutaredoxin
AGAP004164-PA	GSTD1	Glutathione_S-Trfase_N
AGAP004378-PA	GSTD11	Glutathione_S-Trfase_N
AGAP005749-PA	GSTO1	Glutathione_S-Trfase_N
AGAP004802-PA		Glyas_Fos-R_dOase_dom
AGAP006424-PA		Glyco_hydro_1
AGAP005381-PC		Glyco_hydro_catalytic_dom
AGAP002457-PA		Glyco_hydro18cat
AGAP008060-PA	BR2	Glyco_hydro18cat
AGAP002557-PA		GMC_OxRdtase_N
AGAP002568-PA		GNAT_dom
AGAP000949-PA		GOLD
AGAP001064-PA		GOLD

AGAP003553-PA		GOLD
AGAP001502-PA		GroES-like
AGAP004514-PA		GTP_binding_domain
AGAP002341-PA		Haemolymph_juvenile_hormone-bd
AGAP001345-PA		Hemocyanin/hexamerin_mid_dom
AGAP001659-PA		Hemocyanin/hexamerin_mid_dom
AGAP002387-PA		His_Pase_superF_clade-2
AGAP002076-PA		Hsp_70_fam
AGAP004192-PA		Hsp_70_fam
AGAP001424-PA		Hsp90_fam
AGAP000720-PA		Ig_sub2
AGAP003141-PB		Ig_sub2
AGAP004108-PB		Ig_sub2
AGAP000820-PA	CPR125	Insect_cuticle
AGAP001668-PA	CPR5	Insect_cuticle
AGAP002612-PA	CPR7	Insect_cuticle
AGAP002613-PA	CPR8	Insect_cuticle
AGAP002726-PA	CPR9	Insect_cuticle
AGAP003390-PA	CPR124	Insect_cuticle
AGAP005451-PA	CPR11	Insect_cuticle
AGAP005459-PA	CPR16	Insect_cuticle
AGAP006000-PA	CPR25	Insect_cuticle
AGAP006010-PA	CPR105	Insect_cuticle
AGAP006011-PA	CPR31	Insect_cuticle
AGAP001768-PB		Interferon-induced_GILT
AGAP004551-PA		Interferon-induced_GILT
AGAP002728-PA		Isocitrate/isopropylmalate_DH
AGAP004349-PA		KH_dom
AGAP001903-PA		Lactate/malate_DH_N
AGAP004880-PC		Lactate/malate_DH_N
AGAP004609-PA		LDrepeatLR_classA_rpt

AGAP004832-PA		Leu-rich_rpt
AGAP005744-PA	LRIM26	Leu-rich_rpt
AGAP001826-PA	Lp	Lipid_transpt_N
AGAP002592-PA		Lipocln_cytosolic_FA-bd_dom
AGAP002593-PA		Lipocln_cytosolic_FA-bd_dom
AGAP003987-PA		MAM33
AGAP003521-PA		MG_RAP_rcpt_1
AGAP001749-PA		MIR_motif
AGAP001956-PA		ML_dom
AGAP002849-PA		ML_dom
AGAP002850-PA		ML_dom
AGAP002667-PA		Mss4-like
AGAP004877-PB		Myosin_tail
AGAP005118-PA	mRpS17	NA-bd_OB-fold
AGAP007780-PB		NADH_UbQ_OxRdtase_su10
AGAP002889-PA		NADH-UbQ_OxRdtase_b14.5b_su
AGAP001928-PA		NAP_family
AGAP003238-PC		NDRG
AGAP002020-PA		NDUFB8
AGAP004064-PB		NOB1_Zn-bd
AGAP002063-PA		Nop_dom
AGAP004395-PA		Nucleoplasmin
AGAP005129-PA		Nucleoside_phosphorylase_d
AGAP006160-PA		OTU
AGAP004611-PA		Oxoglu/Fe-dep_dioxygenase
AGAP006186-PA		P_typ_ATPase
AGAP002456-PA		P-loop_NTPase
AGAP002879-PA		Pept_cys_AS
AGAP005108-PA		Pept_M24_structural-domain
AGAP005540-PA		Peptidase_C12_UCH
AGAP004809-PA		Peptidase_M1

AGAP002596-PA		Peptidase_M12A
AGAP001791-PA		Peptidase_M13
AGAP000756-PB		Peptidase_M14
AGAP002414-PA		Peptidase_M14
AGAP005558-PA		Peptidase_M16_C
AGAP001245-PA		Peptidase_S1
AGAP001246-PA		Peptidase_S1
AGAP001249-PA		Peptidase_S1
AGAP002422-PA	CLIPD1	Peptidase_S1
AGAP002813-PA	CLIPD6	Peptidase_S1
AGAP003057-PA	CLIPB8	Peptidase_S1
AGAP003246-PA	CLIPB2	Peptidase_S1
AGAP003250-PA	CLIPB4	Peptidase_S1
AGAP003626-PA		Peptidase_S1
AGAP003689-PA	CLIPC7	Peptidase_S1
AGAP004318-PA	CLIPC3	Peptidase_S1
AGAP004719-PA	CLIPC9	Peptidase_S1
AGAP004855-PA	CLIPB13	Peptidase_S1
AGAP005642-PA		Peptidase_S1
AGAP005686-PA		Peptidase_S1
AGAP000994-PA		Peptidase_S28
AGAP003640-PA	SP8905	Peptidase_S28
AGAP004015-PA	SP21408	Peptidase_S28
AGAP001420-PA		PG/BPGM_mutase_AS
AGAP004689-PC		Plexin_repeat
AGAP003134-PA		PPIase_FKBP_dom
AGAP002477-PA		Primosome_PriB/ssb
AGAP004443-PB		Prot_kinase_dom
AGAP001973-PA		Proteasome_asu_N
AGAP004960-PA		Proteasome_asu_N
AGAP005423-PA		Proteasome_asu_N

AGAP004991-PA		Proteasome_sua/b
AGAP005674-PA		PTN/MK_C_dom
AGAP002227-PA		Pyridox_Oxase
AGAP003398-PF		Pyrophosphatase
AGAP005109-PA		QIL1
AGAP004520-PA		Ran_bind_dom
AGAP003742-PB		Reg_chr_condens
AGAP002395-PA	RpL10-2	Ribosomal_L10e
AGAP003025-PC	RpLp2	Ribosomal_L12
AGAP004422-PA	RpL19	Ribosomal_L19/L19e_dom
AGAP001459-PC	RpL17	Ribosomal_L22
AGAP005046-PB	RpL22	Ribosomal_L22e
AGAP002470-PA		Ribosomal_L27
AGAP001408-PA	RpL35	Ribosomal_L29
AGAP003816-PA	RpL30	Ribosomal_L30e
AGAP002754-PA	RpL35a	Ribosomal_L35A
AGAP002921-PB	RpL36	Ribosomal_L36e
AGAP000952-PA	RpL37	Ribosomal_L37e
AGAP002306-PA	RpL4	Ribosomal_L4/L1e
AGAP003538-PA	RpL36a	Ribosomal_L44e
AGAP003770-PA	mRpL51	Ribosomal_L51_mit
AGAP000950-PA		Ribosomal_L9
AGAP002346-PA	RpS14-2	Ribosomal_S11
AGAP004887-PA	RpS17	Ribosomal_S17e
AGAP002407-PA		Ribosomal_S18
AGAP008043-PA	mRpS18	Ribosomal_S18
AGAP001910-PA	RpS3	Ribosomal_S3_C
AGAP003532-PA	RpS3a	Ribosomal_S3Ae
AGAP005061-PB	RpS9	Ribosomal_S4/S9_N
AGAP003768-PB	RpS2	Ribosomal_S5
AGAP002437-PA	RpS8	Ribosomal_S8e

AGAP004441-PA		Ribosome_biogenesis_Nop16
AGAP003663-PA		RNA-helicase_DEAD-box_CS
AGAP001645-PA		RRM_dom
AGAP001930-PB		RRM_dom
AGAP002374-PA		RRM_dom
AGAP002390-PA		RRM_dom
AGAP002654-PA		RRM_dom
AGAP002892-PA		RRM_dom
AGAP003173-PA		RRM_dom
AGAP006365-PA		RRM_dom
AGAP000739-PA	RpS10	S10_plectin_N
AGAP001007-PA		S4_RNA-bd
AGAP007541-PA		SANT/Myb
AGAP003229-PA		SAP_dom
AGAP003016-PA		Saposin-like
AGAP008774-PA		SCO1/SenC
AGAP003091-PA		SCP2_sterol-bd_dom
AGAP004094-PA		SCP2_sterol-bd_dom
AGAP002645-PA		Septin2
AGAP001375-PA	SRPN12	Serpin_fam
AGAP001377-PA	SRPN11	Serpin_fam
AGAP001617-PB		Small_GTPase
AGAP002812-PA		Small_GTPase
AGAP003507-PA		Snf7_fam
AGAP004744-PA		Succ_CoA_synthase_bsu
AGAP003007-PA		SURF6
AGAP002085-PC		SVWC_dom
AGAP007740-PA	RpLp1	T.cruzi_P2-like
AGAP004794-PA		TAG_lipase
AGAP004677-PB		THF_DH/CycHdrlase
AGAP002363-PA		Thioredoxin-like_fold

AGAP003870-PA		THOC7/Mft1
AGAP002948-PA		TIF_eIF_4E
AGAP003119-PA		TIF_eIF-1A
AGAP003052-PA		TPR_1
AGAP002782-PA		TPR-like_helical_dom
AGAP000883-PA		Transl_elong_EF1_G_con
AGAP004235-PA		Transl_elong_EF1B_B/D_CS
AGAP001630-PA	Tpi	Triosephosphate_isomerase
AGAP001797-PD		Tropomyosin
AGAP001797-PI		Tropomyosin
AGAP001799-PA		Tropomyosin
AGAP001053-PB		Troponin
AGAP001053-PE		Troponin
AGAP001053-PG		Troponin
AGAP002350-PB		Troponin
AGAP001701-PA		Ubiquitin-like
AGAP002251-PA		UBQ-conjugat_E2
AGAP005163-PA		UDP_glucos_trans
AGAP004867-PA		V-ATPase_G
AGAP011369-PA		Villin/Gelsolin
AGAP002413-PD		W2_domain
AGAP004418-PA		WD40_repeat
AGAP008075-PC		Znf_CCHC
AGAP000755-PA		Znf_LIM
AGAP003701-PA		Znf_U1
AGAP001023-PF		
AGAP001372-PA		
AGAP001717-PA		
AGAP001718-PA		
AGAP002298-PA		
AGAP002399-PA		

AGAP002619-PA

AGAP002743-PA

AGAP002878-PA

AGAP003043-PB

AGAP003486-PA

AGAP004161-PA

AGAP004237-PA

AGAP004286-PA

AGAP004606-PA

AGAP004618-PA

AGAP004674-PA

AGAP005376-PA

AGAP007777-PA

AGAP008013-PA

Appendix C. Proteins induced after cadmium treatment

Protein stable ID	Gene name	InterPro short description
AGAP001711-PA		4Fe4S-bd_dom
AGAP000272-PA		Adenyl_kinase_AK6
AGAP000235-PC		Beta-thymosin
AGAP000375-PA		Cys_alpha_HP_mot_SF
AGAP000767-PA		Cyt_B5-like_heme/steroid-bd
AGAP001746-PA		FATE/Miff/Tango-11
AGAP000862-PA		Glyco_hydro_31
AGAP002055-PA		Glycoside_Hdrlase_35
AGAP000344-PB	CPR127	Insect_cuticle
AGAP000626-PA		MSP_dom
AGAP001321-PA		NAD(P)-bd_dom
AGAP000170-PA		NADH_UbQ_OxRdtase-like_20kDa
AGAP002170-PA		NuoE-like
AGAP000573-PB	CLIPC4	Peptidase_S1
AGAP001957-PB		PI_transfer
AGAP000308-PA		Proteasome_activ_pa28_N
AGAP001805-PA	RpL13	Ribosomal_L13e
AGAP000655-PA	RpS14-1	Ribosomal_S11
AGAP000399-PA		RRM_dom
AGAP001082-PA		SapA
AGAP001902-PA		Small_GTPase
AGAP002219-PA		Small_GTPase
AGAP000550-PA		Sushi_SCR_CCP_dom
AGAP001325-PA		Thioredoxin-like_fold
AGAP001797-PJ		Tropomyosin
AGAP001799-PB		Tropomyosin
AGAP001219-PA	TUB4A	Tubulin
AGAP001056-PA		UBQ-conjugat_E2

AGAP001823-PA	VATG	V-ATPase_G
AGAP001675-PA		
AGAP001989-PA		

Appendix D: Publications

F1000Research F1000Research 2018, 6:2173 Last updated: 20 JUL 2020

Check for updates

RESEARCH ARTICLE

REVISED Transcriptional responses of *Anopheles gambiae s.s* mosquito larvae to chronic exposure of cadmium heavy metal [version 2; peer review: 1 approved, 1 approved with reservations, 1 not approved]

Catherine N. Muturi [1], Martin K. Rono[2,3], Daniel K. Masiga [4], Francis N. Wachira[5], Richard Ochieng[6], Paul O. Mireji[2,7]

[1]Department of Biochemistry and Molecular Biology, Egerton University, Egerton, Kenya
[2]Centre for Geographic Medicine Research Coast, Kenya Medical Research Institute, Kilifi, Kenya
[3]Pwani University Health and Research Institute, Kilifi, Kenya
[4]Molecular Biology and Biotechnology Unit, International Centre of Insect Physiology and Ecology, Nairobi, Kenya
[5]South Eastern Kenya University, Kitui, Kenya
[6]School of Biological Sciences, University of Nairobi, Nairobi, Kenya
[7]Biotechnology Research Institute, Kenya Agricultural and Livestock Research Organization, Kikuyu, Kenya

v2 **First published:** 22 Dec 2017, **6**:2173
https://doi.org/10.12688/f1000research.13062.1
Latest published: 25 Jun 2018, **6**:2173
https://doi.org/10.12688/f1000research.13062.2

Abstract

Background: *Anopheles gambiae* larvae traditionally thrive in non-polluted environments. We previously documented the presence of the larvae in heavy metal polluted urban aquatic environments and the associated biological cost. The goal of this study was to unravel the molecular dynamics involved in the adaptation of the mosquitoes to the heavy metals.
Methods: Total RNA was extracted from third instar larvae of both cadmium treated populations and untreated control populations. The RNA concentrations were normalized and complementary DNAs were prepared. Then annealing control primer (ACP) technology was applied to establish transcriptional responses in *An. gambiae* larvae following several generational (n=90) chronic exposures to cadmium. Differentially expressed genes were determined by their differential banding patterns on an agarose gel. Gel extraction and purification was then carried out on the DEGs and these were later cloned and sequenced to establish the specific transcripts.
Results: We identified 14 differentially expressed transcripts in response to the cadmium exposure in the larvae. Most (11) of the transcripts were up-regulated in response to the cadmium exposure and were putatively functionally associated with metabolism, transport and protein synthesis processes. The transcripts included ATP-binding cassette transporter, eupolytin, ribosomal RNA, translation initiation factor, THO complex, lysosomal alpha-mannosidase, sodium-independent sulfate anion transporter and myotubularin related protein 2. The down-regulated transcripts were functionally associated with signal transduction and proteolytic activity and included Protein G12, adenylate cyclase and

Open Peer Review

Reviewer Status ? ✓ ✗

	Invited Reviewers		
	1	**2**	**3**
version 2 (revision) 25 Jun 2018		✓ report	✗ report
version 1 22 Dec 2017	? report	? report	

1 **David Essumang**, University of Cape Coast, Cape Coast, Ghana

2 **Shüné V. Oliver**, National Institute for Communicable Diseases, Johannesburg, South Africa

3 **Dick Roelofs**, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

Any reports and responses or comments on the article can be found at the end of the article.

Acta Tropica 198 (2019) 105033

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Cadmium tolerance pathway in *Anopheles gambiae senso stricto*

Martin K. Rono[a,b,*,1], Catherine N. Muturi[c,1], Richard Ochieng[d], Ramadhan Mwakubabanya[c], Francis N. Wachira[e], Joseph Mwangangi[a], Sam Kinyanjui[a,b,f], James Njunge[a], Paul O. Mireji[a,g,*]

[a] KEMRI Centre for Geographic Medicine Research, Coast, P.O. Box 230-80108, Kilifi, Kenya
[b] Pwani University Bioscience Research Centre, Pwani University, P.O. Box 195, Kilifi, Kenya
[c] Department of Biochemistry & Molecular Biology, Egerton University, P.O. Box 536-20115, Egerton, Kenya
[d] International Centre of Insect Physiology and Ecology, P.O. Box 30772-00100, Nairobi, Kenya
[e] South Eastern Kenya University, P.O. Box 170-90200, Kitui, Kenya
[f] Nuffield Department of Medicine, Oxford University
[g] Biotechnology Research Institute- Kenya Agricultural and Livestock Research Organization, P.O. Box 362-00902, Kikuyu, Kenya

ARTICLE INFO

Keywords:
Anopheles gambiae
Cadmium tolerance
Larvae
Proteomics
Immunity

ABSTRACT

Cadmium is one of the widely used heavy metals (HM) in commercial and industrial products and contributes to environmental contamination in an urban setting. In our previous studies, we established that *An. gambiae sensu stricto*, a vector of malaria, had adapted to HM pollutants in nature despite their proclivity for unpolluted aquatic habitats. We further demonstrated that heavy metal tolerance adaptation process impacts a biological cost to the fitness of the mosquito and potentially involves the induction of specific HM-responsive transcripts and proteins. Here we interrogated differential proteomic profiles of the cadmium tolerant vs. naïve strains of *An. gambiae* to shed light on proteomic processes that underpinned biological cost to fitness. We identified a total of 1067 larval proteins and observed significant down-regulation of proteins involved in larval immune responses, energy metabolism, antioxidant enzymes, protein synthesis, and proton transport. Our results suggest that mosquitoes can adjust their biological program through proteome changes to counter HM pollution. Since our study was done in controlled laboratory settings, we acknowledge this may not wholly represent the conditions HM polluted environments. Nevertheless, mosquitoes deploying this strategy have the potential of creating an urban enclave for breeding and thrive and become agents of sporadic malaria epidemics.

1. Introduction

Anopheline mosquitos are the only known vectors of malaria including the most-deadly form caused by *Plasmodium falciparum*. Apart from malaria, some species of *Anopheles* contribute to the transmission of other tropical diseases including; lymphatic filariasis, canine heartworm and O'nyong'nyong fever (Cancrini et al., 2006; Nchoutpouen et al., 2019). Their success in disease transmission is primarily due to the capacity for mosquitoes to exploit different kinds of habitats created by humans, adaptive flexibility that has permitted the mosquito to exploit different larval habitats and colonize a variety of micro and macro environmental conditions throughout the Tropics (Lanzaro et al., 1998; Budiansky, 2002; Roberts et al., 2002; Rozendaal, 1992). Urbanization of Sub-Saharan Africa cities has favored adaptation of anopheline to various xenobiotics and expansion of their niche to habitats polluted by HMs and organic matter contaminants (Awolola et al., 2007; Djouaka et al., 2007; Mukhtar et al., 2003; Sattler et al., 2005; Sibomana, 2002; Mireji et al., 2008). This is contrary to the long-held dogma that *An. gambiae s.l.* exclusively thrive in clean water devoid of pollutants, a common scenario in rural settings (Mireji et al., 2010a). Behavioral plasticity and insecticide resistance have permitted mosquito to either resist insecticides or circumvent them through avoidance. Therefore, limiting the effectiveness of vector control tools such as long-lasting insecticide-treated nets (LLINs) or indoor residual sprays (IRS), thus leading to residual malaria transmission (Rydzanicz et al., 2009; Zhu et al., 2017). To combat malaria transmission especially in urban settings will require novel approaches to be included in the malaria control toolbox (Rydzanicz et al., 2009; Zhu et al., 2017). Understanding the biology of the vectors thriving in polluted urban environments forms the first steps towards designing more effective tools to match the changes in vector dynamics.

Previously, our work demonstrated through multigenerational

* Corresponding authors at: KEMRI Centre for Geographic Medicine Research, Coast, P.O. Box 230-80108, Kilifi, Kenya.
E-mail addresses: mrono@kemri-wellcome.org (M.K. Rono), mireji.paul@gmail.com (P.O. Mireji).
[1] Authors contributed equally.

https://doi.org/10.1016/j.actatropica.2019.05.024
Received 20 January 2019; Received in revised form 16 May 2019; Accepted 18 May 2019
Available online 20 May 2019
0001-706X/

Appendix E: Research Permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **804057**

Date of Issue: **04/November/2019**

RESEARCH LICENSE

This is to Certify that Ms.. Catherine Ngambi of Egerton University, has been licensed to conduct research in Nairobi on the topic: IMPACT OF HEAVY METAL TOLERANCE ON GENE EXPRESSION, GENOME MUTATION AND INFECTIVITY IN PLASMODIUM OF ANOPHELES GAMBIAE MOSQUITO VECTOR OF MALARIA for the period ending : 04/November/2020.

License No: **NACOSTI/P/19/2391**

804057

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

THE SCIENCE, TECHNOLOGY AND INNOVATION ACT, 2013

The Grant of Research Licenses is Guided by the Science, Technology and Innovation (Research Licensing) Regulations, 2014

CONDITIONS

1. The License is valid for the proposed research, location and specified period
2. The License any rights thereunder are non-transferable
3. The Licensee shall inform the relevant County Director of Education, County Commissioner and County Governor before commencement of the research
4. Excavation, filming and collection of specimens are subject to further necessary clearence from relevant Government Agencies
5. The License does not give authority to tranfer research materials
6. NACOSTI may monitor and evaluate the licensed research project
7. The Licensee shall submit one hard copy and upload a soft copy of their final report (thesis) within one of completion of the research
8. NACOSTI reserves the right to modify the conditions of the License including cancellation without prior notice

National Commission for Science, Technology and Innovation
off Waiyaki Way, Upper Kabete,
P. O. Box 30623, 00100 Nairobi, KENYA
Land line: 020 4007000, 020 2241349, 020 3310571, 020 8001077
Mobile: 0713 788 787 / 0735 404 245
E-mail: dg@nacosti.go.ke / registry@nacosti.go.ke
Website: www.nacosti.go.ke