RESPONSES OF MOSQUITOES TO HEAVY METALS

MIREJI PAUL ODHIAMBO

BSc. (Biochemistry, Chemistry), Egerton University, Njoro, Kenya.
MSc. (Tropical Entomology), University of Zimbabwe, Harare, Zimbabwe.

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Declaration

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

Paul .O. Mireji

Signed: _____ Date: 7/28/06

We confirm that the work reported in this thesis was carried out by the candidate and has been submitted with our approval as supervisors

Dr. Eucharia U. Kenya.

Department of Biochemistry and Biotechnology, Kenyatta University, Kenya

Signed: _____ Date: 12/10/2006

Dr. Hudson Nyambaka

Department of Chemistry, Kenyatta University, Kenya

Signed: _____ Date: 11/10/06

Dr. Charles Mbogo
KEMRI-CGMRC-Kilifi, Kenya

Signed: _____ Date: 6/10/06

Dr. John C. Beier

Department of Epidemiology and Public Health, University of Miami, School of Medicine, Miami, Florida, USA.

Signed: _____ Date: 9/28/06

Dedication

To my loving wife Mrs Lucia Akinyi Odhiambo

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Glossary of Abbreviations

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DTT	Dithithreitol
GPS	Global Positioning System
IEF	Isoelectric Focusing
KDa	Kilo Dalton
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS-PAGE	Sodium Dedosyl Sulphate Polyacrilamide Gel Electrophoresis
WHO	World Health Organization
2D-PAGE	Two Dimensional Polyacrilamide Gel Electrophoresis

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Abstract

Investigations were conducted to determine the influence of heavy metals on urban mosquito populations spatial distribution and composition and *Anopheles gambiae sensu stricto* fitness. Differential induction of proteins as well as expression of metallothionein, alpha tubulin gene expressions in third instar *An. gambiae s.s* larvae in response to heavy metals selection was also investigated. Heavy metal concentrations and mosquito larvae species composition in larval habitats of urban Kisumu and Malindi, Kenya were determined by atomic absorption spectroscopy (AAS) and taxonomic keys respectively. A susceptible strain of *An. gambiae s.s* third intar larvae was separately placed under selection pressure with cadmium, copper and lead at LC_{30} and control through five generations. Egg hatchability, fecundity and survivorship of the larvae and adults were monitored in the sixth generation in the absence of heavy metal selecting agent. First, third and fifth generation selection survivors were screened for expression of the metallothionein and alpha tubulin genes by semi quantitative RT PCR and for differentially expression proteins by 2D gel electrophoresis. Manganese and iron were the most prevalent heavy metals in larval habitats in urban Kisumu and Malindi respectively. Concentrations of most were above WHO acceptable limits for drinking water. Copper and lead concentrations significantly influenced presence of *Ae. Aegypti,* while presence of *An. gambiae* was affected by lead concentrations. Resistance to cadmium, copper and lead selection increased 13, 11 and 78 folds respectively between first and fifth generation. There was significant reduction in egg viability, larvae survivorship, pupation, magnitude of adult emergence, fecundity, and delay in pupation in the heavy metals selected populations relative to control. The innate rate of increase and mean generation time were significantly lower in heavy metal selected populations than control. Instantaneous birth rate was also significantly lower in all but lead selected populations, than control. Population doubling time was significantly higher in the heavy metals selected than in the non-selected control populations.Expression of metallothionein was significantly higher in cadmium and copper selected than control populations in third and fifth generations respectively.Expression of alpha tubulin was significantly higher in cadmium selected than in control populations in fifth generation. Most differentially expressed protein spots were acidic and of low molecular weight among all metals and generations. Type of heavy metals and generation (selection pressure) were main indicators of differential expression magnitude. Mosquitos that traditionally exclusively proliferate in clean water like *An. gambiae* seems capable of expanding their niche into polluted habitats and other hostile environments, but at a considerable biological costs. Semi quantitative RT PCR was sufficiently sensitive to detect heavy metals resistance/ adaptation in *An. gambiae s.s* larvae in a metal, gene and selection pressure specific manner. Both genes display both qualitative and quantitative potential application in monitoring both *An. gambiae s.s* adaptation status to heavy metals selection as well as biomonitoring heavy metals pollution through larval habitat. 2D gel electrophoresis also appears sufficiently sensitive to detect heavy-metals and generation specific responsive genes in *An. gambiae s.s* larvae and can also be used to biomonitor heavy metals environmental anopheles adaptation to pollution. This process would greatly be facilitated by characterization of the spots by mass spectrometry and molecular functional analysis of the spots against *An. gambiae* protein database.

Chapter 1

Introduction and Literature Review

1.1 Urbanization and Pollution in Africa

The last century has witnessed the emergence and growth of urban centers in Africa. The urbanization has been accompanied by rapid population growth, expansion of industrial activities and lack of environmental regulation, and associated with poorly planned and drained cities, leading to environmental pollution levels in excess of natural loads (Biney *et al.,* 1994). Available information on heavy metal prevalence in cities and water bodies in Africa indicate that enrichment of cadmium, cobalt, copper, chromium, iron, manganese, nickel, lead and zinc in Lagos lagoon Nigeria, originate from land-based urban and industrial anthropogenic sources (Okoye *et al.,* 1991). Additionally, higher levels of lead in sediments than in water have been reported in Ibadan, Nigeria, with the highest levels coinciding with the areas of high traffic density (Mombeshora *et al.,* 1983). Increased lead levels in Lake Victoria, Kenya, have also been observed and attributed to increased shipping traffic and associated problems, car washing and discharges from local industries (Wandiga and Onyari 1987; Onyari and Wandiga 1989). Lake Victoria receives domestic and industrial discharges from surrounding cities, including Kisumu. High levels of arsenate, cadmium, copper, manganese, lead, zinc and mercury have also been detected in Hartbeespoort dam which receives industrial and municipal waters from Johannesburg, South Africa (Greichus *et al.,* 1977).

Chang and Cockerham, (1994) have documented that urban heavy metal environmental pollution has both natural and anthropogenic origin, and is stable and persistent, with

fossil fuel combustion releasing about 20 toxicologically important heavy metals, which affecting remote areas away from their source while other sources include industrial products, used industrial materials and fertilizer.

1.2 Urbanization and Mosquito Ecology

The Urbanization and accompanying rapid human population and pollution increases has significantly affected mosquito ecology (Chinery, 1984) probably through reduction of clean larval habitats, favorable for anopheline survival. The urbanization initially supports mosquito proliferation by presenting favorable aquatic habitats while subsequently depressing mosquito population, especially anopheline, by reducing favorable habitats through processes such as aquatic pollution (Trape and Zoulani, 1987a). Urbanization has therefore, led to significant reduction in mosquito species diversity in urban African, including in towns such as Accra and Tema, Ghana (Chinery, 1995), Burkina Faso, Benin (Coluzzi, 1993), Kinshasha, DRC (Coene, 1993) and Brazzaville, Congo (Trape and Zoulani, 1987a).

Additionally, urbanization has also adversely affected anopheline daily survival and life expectancy (Coluzzi, 1993; Chinery, 1984), including in towns such as Kinshasa, DRC (Coene, 1993), and Accra and Tema, Ghana (Chinery, 1995). These phenomena have given rise to low malaria transmission in the urban centers, compared to peri-urban and rural centers (Robert *et al.,* 2002). These ecological effects have been attributed to pollution of the larval habitats among other factors (Trape and Zoulani 1987b). Most culicine mosquitoes, including *Culex quinquefaciatus* have adapted to polluted urban

larval habitats (Subra, 1981), while most anophelines have been relegated to peri-urban regions, as observed in Kinshasha, DRC (Coene, 1993) and Brazzaville, Congo (Trape and Zoulani, 1987b).

However, possible adaptation of anopheline mosquitoes to environmental pollution has been suggested by recent observations of anopheline larvae in polluted urban larval habitats, contrary to the dogma that anophelines can only be maintained in relatively clean water habitats. Polluted drains containing domestic wastewater diluted by rain have been observed supporting anophelines in Kinshasa, Zaire (Coene, 1993). Similarly, *An. gambiae* has been observed in polluted man-made aquatic habitats in Accra and Tema, Ghana (Chinery, 1984; Chinery, 1995).

The anopheline adaptation can be attributed to their niche expansion into polluted habitats in which intraspecific competition is less severe than in the limited number of habitats. This phenomenon has been demonstrated in *Drosophila melanogaster* adaptation to cadmium-contaminated environment (Bolnick, 2001) and is further supported by the adaptability of *An. gambiae* to environmental changes. Coluzzi *et al.*, (1979) suggest that in southern Nigeria, urban isolates of *An. arabiensis* probably originated through passive adaptation to city environments. In Mali, *An. gambiae* Mopti has adapted to larval habitats present in dry seasons and normally linked to human activity, unlike *An. arabiensis* and other taxa of *An. gambiae* complex which are more or less dependent on rain-created larval habitats (Coluzzi, 1994; Toure *et al.*, 1998).

1.3 Biological Response of Insects to Pollution

The maintenance of anophelines in polluted urban environments, like other aquatic insects, can be attributable to tolerance of their aquatic developmental stages to environmental pollutants, including heavy metals. Chronic exposure of aquatic insect populations such as *Chironomus tentans* (Wentsel *et al.,* 1978) and *Baetis thermicus* (Suzuki *et al.,* 1988), to heavy metals has been shown to increase their tolerance to heavy metals (Krantzberg and Stokes, 1990; Hare, 1992; Clements and Kiffney, 1994). Heavy metals are frequently detected as ground water contaminants (Clements and Kifney, 1994) in urban environments. The metals originate directly from mines, smelters and industries and are transported in the atmosphere (Nriagu and Pacyna, 1988), thereby affecting water bodies distant from local source.

The tolerance to heavy metal can be attributed to exclusion and/or active metal excretion in aquatic insects. This phenomenon can be indicated by poor correlations between environmental and physiological heavy metal levels in aquatic insects (Klerks and Weis, 1987; Krantzberg and Stokes, 1990). The tolerance can also be due to bioaccumulation of heavy metal and is responsible for *B. thermicus* tolerance to cadmium and copper exposure (Suzuki *et al.,* 1988).

Aquatic insect tolerance to heavy metals varies with different genotypes and strains within a species (Hare, 1992; Clements and Kifney, 1994), and occurs at both population and individual genetic response levels (Beaty *et al.,* 1998). The heavy metal tolerance can therefore affect insect species distribution and abundance (Hare, 1992). The tolerance can

be both metal and concentration dependent as shown in *Aedes aegypti* larvae responses to cadmium and copper (Rayms-Keller *et al.*, 1998). Heavy metal exposure at critical stages of aquatic insect growth can significantly affect the insect fitness (Hare, 1992; Rayms-Keller *et al.*, 1998). It has been suggested that metallic scum in water prevents culicine larvae metamorphosis and pupation (Wagbatsoma and Ogbeide, 1995). However, insects adapted to heavy metals display depressed fecundity and survivorship on exposure to unpolluted environments (Shirley and Sibly, 1999; Belfiore and Anderson, 2001).

1.4 Cellular Responses to Heavy Metals

Heavy metals such as cadmium and lead are not known to be natural components of any biological system. Their relative absence from the environment as a free metal ions are mainly due to their sequestration as ores. It can be hypothesized that cadmium and lead were not adopted during evolution as important component in living systems because they were relatively absent in cellular milieu, suggesting that no selective pressure was applied to proteins to be able to distinguish the metals from biologically important and intracellularly present metals such as calcium and zinc (Bouton and Pevsner, 2000).

Heavy metals, such as cadmium, affect many cellular functions by modification of cell growth and metabolism at sub-lethal and marginally lethal levels, and apoptosis and necrosis at lethal levels. It is proposed that cadmium induces cellular damage through oxidative stress (Stohs *et al.*, 2001) by binding to reduced cystein residues and generating reactive oxygen species, and by lowering intracellular glutathion levels (Hussain *et al.*,

1987; Manca *et al.*, 1991; Chin and Templeton, 1993; Koizumi and Li, 1992; Abe *et al.*, 1994; Acan and Tezcan, 1995).

The reactive oxygen species are mainly composed of superoxide ion, hydroxyl radicals, and hydrogen peroxide (Stohs *et al.*, 2001). The oxidative stress is accomplished through lipid peroxidation, modulation of intracellular oxidized states, production of nuclear factor-kappaB, and induction of DNA damage (Stohs *et al.*, 2001). The reactive oxygen species also induce membrane damage, alter gene expression, induce apoptosis (Stohs *et al.*, 2001) and delay DNA synthesis (Enger *et al.*,1987). It also disrupts intracellular sulfhydryl homeostasis (Beyersmann and Hechtenberg, 1997) and induces tumors (Abshire *et al.*, 1996). Cadmium increases lipid peroxidation and other oxidative stress factors by inhibiting glutathione reductase (Acan and Tezcan, 1995; Koizumi and Li, 1992) and superoxide dismutase (Hussain *et al.*, 1987), reducing catalase and increasing glucose-6-phosphate dehydrogenase and glutathione peroxidase activities (Salovsky *et al.*, 1992). Effects of cadmium with potential genetic consequence also occur in the inhibition of DNA repair (Hartwig *et al.*, 1996), DNA polymerases, and RNA transcription and translation (Beyersmann and Hechtenberg, 1997).

Cells counteract the damage induced by heavy metals by transcription of genes encoding for defense and repair proteins. These proteins chelate the metal to prevent further damage, remove reactive oxygen species, repair membrane and DNA damage and re-nature or degrade unfolded proteins (Liao and Freedman, 1998). The proteins include heat shock proteins (Abe *et al.*, 1994) and metallothionein (Shimizu *et al.*,1997). The

cells also activate proto-oncogenes, responsible for cell proliferation and/or apoptosis, by transcriptional activation of early response genes including - *c-jun, c-fos, c-myc* and erg-1 in various cells (Matsuoka and Call, 1995; Jin and Ringertz, 1990; Hechtenberg *et al.,* 1996; Abshire *et al.,* 1996; Shimizu *et al.,* 1997; Wang and Templeton, 1998). In case of cytotoxicity, apoptosis is induced as mode of elimination of damaged cells (Habeebu *et al.,* 1998). Cadmium elicits anti-tumor effect (Terracio and Nachtigal, 1988; Abshire *et al.,* 1996) by induction of p53 tumor suppressor gene (Stohs *et al.,* 2001). Cadmium inhibits and stimulates early and late epidermal growth factor-induced DNA synthesis (Enger *et al.,* 1987; Tang and Enger, 1993).

Cadmium evokes intracellular effects via a reversible interaction with an external receptor on the cell surface as observed in human skin fibroblasts (Smith *et al.,* 1989). Cadmium can enter through receptor operated calcium channels (Blazka and Shaikh, 1991), passive transport (Shaikh *et al.,* 1995), SH-ligand containing transport processes associated with the uptake of zinc, copper and iron and voltage-sensitive calcium channels (Hinkle *et al.,* 1994).

Heavy metals such as cadmium and lead modulate activities of complex signal transduction pathways that in turn influence expression of a myriad of genes. The pathways include those mediated by protein kinase C (PKC), calcium, zinc, cAMP dependent protein kinase, calmodulin and mitogen activated protein kinases (Templeton *et al.,* 1998; Beyersmann and Hechtenberg, 1997; Bressler *et al.,* 1999; Goldstein, 1993; Son *et al.,* 2001). They also activate cadmodulin-mediated pathway by substitution of

calcium in binding to calcium binding sites (Behra and Gall, 1991; Goldstein and Ar, 1983). Lead has also been shown to improperly substitute for zinc in aminolevulinic acid dehydrogenase (Warren *et al.,* 1998). Carbonic anhydrase, a zinc dependent enzyme is also inhibited by lead (Goering, 1993). Although lead is effectively able to compete with zinc to bind to zinc sites, lead does not induce the proper structural conformation once bound (Hanas *et al.,* 1999), leading to aberrant expression of Sp1 target gene such as myelin basic protein and proteolipid protein (Zawia and Harry, 1996). Protein kinase C activation may also be induced through the cadmium-induced oxidative stress (Bagchi *et al.,* 1997; Stohs *et al.,* 2001).

Upon activation by cadmium, PKC has been observed to mediate induction of early response genes (Tang and Enger, 1993; Matsuoka and Call, 1995; Templeton *et al.,* 1998). Induction of these proto-oncogenes is largely independent of mitogen activated protein kinase system (Beyersmann and Hechtenberg, 1997). Lead has also been observed to enhance AP-1 DNA binding through a PKC mediated pathway (Chakraborti *et al.,* 1999), and induction of immediate early genes expression through activation of PKC (Kim *et al.,* 2000).

Cadmium may also activate transcription through specific metal-responsive upstream regulatory elements found in the promoter regions of cadmium responsive genes, including metal responsive element (MRE) sequences found in most metallotheonein genes (Stuart *et al.,* 1984). Glutathione is an effective oxygen radical scavenger (Yu, 1994) and provides the first line of defense against cadmium before metalotheonein

induction (Singhal *et al.,* 1987) or apoptosis (Son *et al.,* 2001). Glutathione has also been implicated in acquired cadmium resistance in human lung carcinoma A549 cells attributed to enhanced gamma-glutamylcysteine synthetase expression (Hatcher *et al.,* 1995). Metallothionein and alpha tubulin induction in *Drosophila melanogaster* larvae and *Chironomus tentans* midgut epithelia, respectively, have also been observed upon exposure to sub-lethal cadmium and copper concentrations (Maroni and Watson, 1985; Mattingly *et al.,* 2001) and may be involved in counteracting toxicity of respective metal. However, when the toxicological insults of heavy metals cannot be counteracted, toxicity ensues (Tiffany-Castiglioni, 1993). Chronic cadmium exposure condition *Ae. aegypti* survivorship (Rayms-Keller *et al.,*1998) by compromising peritrophic matrix integrity, despite inducing of intestinal mucin (Rayms-Keller *et al.,* 2000).

Excessive environmental levels of heavy metals can therefore significantly affect mosquito aquatic biota since the metals are toxic, being easily incorporated into biological molecules. They exert their toxicity by displacing essential metals of a lower binding power in biologically active molecules or by acting as non-competitive inhibitors of enzymes. Conversely, mosquito populations may adapt to the heavy metal contaminants probably through development of physiological or molecular mechanisms that counteract the toxicity. However, such mechanisms have not been elucidated, nor has the biological cost of such adaptation been established. The mechanisms may involve expression of specific metal responsive genes that counteract heavy metal toxicity, with the cost of adaptation expressed on its impact on mosquito composition, distribution and fitness.

1.5 Adaptation and Evolution of Resistance to Xenobiotics and Novel Environments

Application of xenobiotics for control of pathogenic organisms and pests has often led to the rapid spread of alleles conferring resistance (Palumbi, 2001), which induce resistance genes that mediate toxicant metabolism or alter toxicant targets (Kalow, 1997). These genes are generally formed by modification of a pre-existing gene and are thus rarely *de novo* synthesized gene (Dujon *et al.*, 2004). This modification often involves substitution of alleles imparting major effect (Roush and McKenzie, 1987; Denholm and Rowland, 1992; McKenzie and Batterham, 1994). These alleles are associated with extremely high selection intensities, often occurring in field exposures (McKenzie *et al.*, 1992; McKenzie and Batterham 1994; McKenzie, 2000) while polygenic control of resistance is mainly associated with lower intensity of selection associated with laboratory selection (McKenzie and Batterham, 1994). At proteomic level, enzyme induction mediates moderate degrees of resistance, and is reversible and non-inheritable (Okey, 1992). On the other hand, high degree of resistance is attributable to proliferation of initial rare, naturally insensitive individual survivors of acute and chronic insecticide exposure (Kalow, 1997). This phenomenon is attributable to pre-existing resistant genotype in the population and low frequencies, a fact underscored by the fact that most pharmacogenetic variants within a population are pre-adaptive (Kalow, 1997). It is in this respect that selection should ideally be conducted with moderate to strong selection pressure and initiated with large populations and with genetic diversity representative of field populations (Tabashnik, 1992).

The presence of refuges significantly influence the evolution of resistance. Refuges delay evolution of resistance and are more effective when selection is more intense than when selection is weaker (Groeters and Tabashnik, 2000). They display their most effect where resistance is functionally recessive (Georghiou and Taylor 1977, Tabashnik and Croft 1982; Groeters and Tabashnik, 2000). While resistance is effected by many genes, distribution of effects across loci is not usually uniform, with one or a few loci generally accounting for most of the resistance (Groeters and Tabashnik, 2000). In this respect, location and understanding of the major loci can facilitate elucidation of the mechanism of resistance and improve the ability to track and delay the evolution of resistance (Groeters and Tabashnik, 2000).

Genetic variation of ecologically important traits critically influences the ability of populations to adaptively evolve. This is paramount to maintenance genetic variation for reproductive fitness, and fitness levels themselves. These parameters may be influenced by critical variables including long term effective population size (Li, 1978), rate and effect of spontaneous mutations (Lynch and Walsh 1998; Fry *et al.*,1999; Shaw *et al.*, 2000; Zeyl *et al.*, 2001), dominance patterns, epistasis, and pleiotropy of mutations (Johnston and Schoen 1995; Ferna´ndez and Lo´pez-Fanjul 1996) and strength and nature of selection acting on those mutations (Lynch and Walsh., 1998). Thus smaller populations should have lower fitness because of increased inbreeding depression (Charlesworth and Charlesworth 1987), lower efficiency in eliminating deleterious alleles (Li 1978) and presence of fewer beneficial mutations (Kimura 1983). However it has

paradoxically been shown that quantitative traits habitability can remain high, or even increase, despite reduction of the population (Bryant *et al.*, 1986).

Prior environments experienced by populations can greatly influence fitness in a novel environment and the population's ability to adapt to a novel or changing environment (Reed *et al.*, 2003). Differential selection caused by a varying environment is theoretically capable of maintaining genetic polymorphisms (Felsenstein 1976; Gillespie and Turelli 1989) and there is empirical evidence linking variable selection or habitat heterogeneity with increases in genetic variation (Gram and Sork 2001). Besides the heterogeneity of the environment, the level of stress an environment imposes can affect fitness levels and genetic variability for fitness (Fowler and Whitlock 1999, 2001). Genes that control generalized stress resistance, such as heat shock proteins, are important in allowing organisms to adapt to novel or changing environments. Variable environment maintain greater levels of genetic variation for fitness with the populations with the lowest initial fitness experiencing the greatest selection and has the smallest response to the selection (Reed *et al.*, 2003). Reed *et al.*, (2003) have also documented that populations inbred in a variable environment are more adaptable than those inbred in a constant environment. They have also indicated that populations adapted to a prior stressful environment have greater fitness when reared in a novel stress than those less adapted to stress and that inbred populations have lower fitness and are less adaptable than the original out bred population.

It has also been shown that adaptation to one stress leads to long term higher fitness levels in the presence of an unrelated stress (Reed *et al.,* 2003), suggesting that populations adapted to a particular stress may more easily invade other extreme environments, even if the two stresses are unrelated (Reed *et al.,* 2003). In this respect they can also conduct niche construction by modifying local resource distributions, influencing both their ecosystems and the evolution of traits whose fitness depends on such alterable sources of natural selection in environments (Laland *et al.,* 1999). This can be a potent evolutionary agent by generating selection that leads to the fixation of otherwise deleterious alleles, supporting stable polymorphisms where none are expected, eliminating what would otherwise be stable polymorphisms, and generating unusual evolutionary dynamics (Laland *et al.,* 1999). The rate of fitness increase during adaptation depends both on current fitness and size of the random mutations produced. Given the same size of mutations and the same total strength of natural selection, complex organisms cannot adapt as quickly as simpler ones (Orr, 2000). Adaptation is characterized by an initial rapid rise in fitness, followed by a gradual slowing as the population approaches the phenotypic optimum (Orr, 2000).

1.6 Cost of Adaptation/Resistance to Xenobiotics

Adaptations conferring resistance to xenobiotics are often costly to the organism's fitness in the absence of the selecting agent (Orr 1998), Unless these costs are compensated for by secondary mutations (Levin *et al.,* 2000), the frequency of resistant individuals is expected to decline in untreated environments as they are displaced by sensitive individuals having a greater reproductive success and superior rates of population growth

(Agnew *et al.*, 2004). This loss of stress resistance is generally rapid and common across various taxa, the rate and the magnitude of which is proportional to the costs of resistance (Agnew *et al.*, 2004). This phenomenon is probably attributable to a cost associated with maintaining genes for stress resistance or to an increased frequency of deleterious mutations currently segregating in the population (Mukai *et al.*, 1972; Fry 2001; Zeyl *et al.*, 2001). However, this evolutionary dynamic is open to modification by other sources of selection acting on the relative fitness of susceptible and resistant individuals (Agnew *et al.*, 2004). Although many mutations and their functional role in conferring resistance to xenobiotics have been identified (Glass *et al.*, 1986; Weill *et al.*, 2003), the actual costs they impose is often unclear. This situation is attributable to difficulties in isolating variation in fitness due to the possession of a resistance mutation from that due to variation in the genetic background in which they are expressed (McKenzie *et al.*, 1982; Schrag *et al.*, 1997), and from variation in the environmental conditions in which these costs are measured (McKenzie 1994). Parasitism has also been associated with compromising the fitness of infected mosquitoes (Agnew *et al.*, 2004).

However, the cost of resistance can be compensated for through compensatory adaptation, which includes both resistance mutations and deleterious mutations in general. For example, Björkholm *et al.*, (2001) established that clarithromycin resistance in *Helicobacter pylori* confer a biological cost, as measured by a decreased competitive ability of the resistant mutants in mice, which could be reduced by stabilizing resistant bacteria in a population. They suggested that was attributable to development of compensatory mutation after the resistance mutation occurred, resulting in a strain with

increased fitness. They therefore postulated that there is probably a stepwise selection from sensitive to resistant to compensated mutant during pre- and post selection regiments.

Thus, compensatory mutations provide an opportunity to study the origin and maintenance of gene interactions (epistasis) in adaptive evolution (Wijngaarden *et al.,* 2004). Compensating second site mutations make a return to the susceptible state unlikely, as back mutations at either the resistance locus or the compensatory locus result in genotypes with a lower fitness than both the uncompensated susceptible and the compensated resistant genotypes (Wijngaarden *et al.,* 2005). Hence, it shows how two loci can become parts of a group of co-adapted genes that effectively prevent reverse evolution (Teoto´nio and Rose 2001). Reed *et al.,* (2003) suggest that large populations reintroduced to a stressful environment can develop to normal levels due to their genetic diversity while this may not be the case with small captive population due to inbreeding depression and loss of genetic diversity.

1.7 Justification

It is anticipated that levels of pollution will increase with industrialization of African. This development is bound to increase pollution levels to those observed in industrialized nations. Such increases in environmental pollution will naturally affect the levels of pollution in mosquito habitats in urban areas. This in turn will influence mosquito proliferation and hence the epidemiology of the various mosquito-borne diseases such as malaria, since the proliferation of these vectors is affected significantly by the quality of water in their habitats. Urbanization, accompanying industrialization has also been coupled with poor drainage and

planning of African cities, further aggravating the situation. Urban pollutants include both heavy metals and non-metals. However, heavy metal pollution, especially from petrochemical combustion is more widespread, anthropogenic and ubiquitous. It is also desirable to understand the molecular mechanism through which the mosquito would counteract or adapt to such toxicants. It is prudent that establishing baseline levels of heavy metals in larval habitats in urban Africa and their influence on mosquito ecology should precede such investigation. Anophelines are known to proliferate exclusively in non-polluted larval habitats. However, recent observations have been made of anopheline mosquito proliferation in polluted larval habitats in some cities in sub-Sahara Africa, suggesting that they are developing resistance to heavy metals. It is therefore necessary to establish how common industrial pollutants, such as heavy metals, influence mosquito ecology, biological fitness, and establish the underlying molecular mechanism..

1.8 Hypotheses

1. Heavy metals in larval habitats influence mosquito species composition and spatial distribution.
2. *Anopheles gambiae* fitness is a function of heavy metal concentrations in their aquatic larval habitats.
3. Mosquito tolerance to heavy metals is mediated through specific molecular process(es).

1.9 Objectives

1.9.1 General Objective

The general objective is to study biological responses of anopheline mosquitoes to heavy metals and other environmental contaminants.

1.9.2 Specific Objectives

1. To determine how mosquito species composition and spatial distribution patterns are affected by heavy metals in aquatic larval habitats in urban Kisumu and Malindi.
2. To evaluate effect of heavy metals on *An. gambiae* fitness
3. To identify molecular mechanisms by which *An. gambiae* respond to heavy metals.

Chapter 2

Materials and Methods

2.1 Field studies

Assessment was conducted on heavy metals prevalence in mosquito larval habitats in urban Kenya, with a focus on two towns, Kisumu and Malindi , and how the prevalence has influenced mosquito species and composition.

2.1.1 Study Areas

Kisumu (00°06'S 034°45'E) is located on the shores of Lake Victoria, Kenya, with a population of about 329,000 (GOK, 1999). The town is located between 1,075 and 1,150 m above sea level with mean daily minimum and maximum temperatures of 18 and 30 °C, respectively. Kisumu experiences long and short rain seasons from March to May and September to December, respectively. The mean annual rainfall levels are between 1,000 and 1,500 mm. Commercial and industrial activities in urban Kisumu include ballast, bolt and nut production, sand harvesting, food processing, rebounding and re-facing of motor vehicle parts, and farm machinery assembly. Many rivers in Kisumu are polluted with agrochemicals and pesticides from the catchment areas (Government of Kenya, 1999). Anopheline species composition in Kisumu region includes *An. arabiensis, An. funestus* and *An. gambiae* (Githeko *et al.,* 1993).

Malindi (03°21'S 40°10'E) is located on the shore of the Indian Ocean in Kenya with a population of about 120,000 (Government of Kenya, 1999). The town is located between 0 and 50 meters above sea level with mean daily minimum and maximum temperatures of 22 and 30°C, respectively. Malindi experiences long and short rainy seasons from

April to June and October to November, respectively. The mean annual rainfall levels are between 750 and 1,200 mm. Malindi has high groundwater levels, leading to formation of dams during the rainy seasons (Government of Kenya, 1999). Commercial activities in Malindi include palm oil extraction, salt processing and harvesting coral and galena stone mining. Other activities include agriculture, fisheries, tourism and trade. Anopheline composition in the coast of Kenya includes *An. arabiensis, An. funestus*, *An. gambiae* and *An. merus* (Mbogo *et al.*, 1995).

2.1.2 Sampling Design and Procedure

A cross-sectional survey of cadmium, chromium, copper, iron, lead, manganese and zinc prevalence in larval habitats in Kisumu and Malindi was conducted by collecting duplicate samples of water and sediment by random sampling of larval habitats in the two cities. Mosquito larvae were also sampled from each selected habitat. The survey was conducted from 17 to 24 June and 7 to 15 July 2002 in Malindi and Kisumu respectively.

Previously defined stratification of the two cities based on levels of planning and drainage (Keating *et al.*, 2003) were applied in sampling. The towns had been stratified into four categories, namely well-drained well-planned; well-drained, poorly-planned; poorly-drained, well-planned and poorly-drained poorly-planned areas. Each of the regions in the two towns had been demarcated into 270 x 270 m grid cells (Appendices 6.1-6.4). Kisumu and Malindi comprised of 317 and 244 grid cells, respectively. The number of grid cells selected for sampling in each city was proportional to the size of the strata in the entire city. Forty and 55 grid cells were sampled in urban Kisumu and

Malindi, respectively. Well-drained, well-planned; poorly drained, well planned; well-drained poorly planned and poorly drained, poorly-planned strata were represented by 14, 5, 2 and 19 grid cells, respectively, in Kisumu, and 15, 9, 4 and 27 grid cells, respectively, in Malindi.

One larval habitat was randomly selected within each selected grid cell for sampling by sampling the first water body from the center of the grid cell in a direction randomly identified. All water bodies encountered were considered potential larval habitats. Water samples (250 ml) were collected in plastic bottles and immediately acidified to pH < 2.0, by adding 1 ml of analytical grade concentrated HNO_3, to prevent heavy metal adsorption to the container walls and inhibit the activity of micro-organisms which might cause changes in trace metal levels in the water samples. Bottom sediment samples were collected by a sampling corer and placed in plastic bags. Both samples were collected in two replicates and immediately stored at 4 ^{0}C until when they were analyzed. Larval samples were also collected from the habitats using a standard mosquito-sampling dipper (350 ml), and immediately preserved in absolute ethanol until when they were required for identification. Characterizations of the mosquito specimens were done using morphological taxonomic keys (Gillett, 1972) into *Anopheles gambiae sensu lato* (hence forth simply referred to as *An. gambiae* in the field studies component of this thesis), *Anopheles funestus, Culex quenquifasciatus,* and *Aedes aegypti.* The geographical co-ordinates of each habitat were determined using Garmin® hand held GPS unit. Degree of permanence of the habitats was also noted. A habitat was considered temporary, semi-

permanent and permanent if water body seemed capable of drying within a month, had seasonal presence lasting up to six months and perennial respectively.

2.1.3 Preparation of Water and Soil Samples for Heavy Metal Determination

Water samples were subjected to acid digestion since the samples contain suspended solid particles. Briefly, 100 ml of each sample solution was placed in 50 ml of nitric acid (Analar grade) (1:1) and the solution digested by gently heating until the volume reduced to a third (50 ml). The solution was allowed to cool down to room temperature before 20 ml of HCl (1:1) was added and heated gently for another 10 min to complete the digestion. The solution was filtered, and the filtrate volume adjusted to 100 ml with distilled water and stored in plastic bottles until when required for analysis.

Sediment samples were oven dried at 50 °C for 12 h and ground using a pestle and mortar. The powder was sieved through a 50-75 μm screen. A sediment sample (1g) was placed in a Teflon beaker, acidified with 2 ml of concentrated HNO_3 and evaporated to dryness at 80 °C. It was further digested in concentrated HNO_3, $HClO_4$ and HF (30:2:10 by volume) mixture at 60 °C and evaporated to dryness with temperature increased gradually to 120 °C to remove $HClO_4$. Digestion was completed by addition of H_2O_2 and the solutions were filtered into plastic containers. The residue was rinsed with 0.1 M HCI and the filtrate volume adjusted to 100 ml with distilled water. Blank digests of the filter paper were also prepared and were used, in both water and water sample preparations, to account for matrix effects, hence aid in accurate determination of the heavy metals in the samples.

2.1.4 Heavy Metal Determination

Quantitative and qualitative determination of cadmium, chromium, copper, iron, lead, manganese and zinc heavy metals in the water and soil samples was conducted on Buck Scientific 210VGP Atomic Absorption spectrophotometer according to the manufacturer's instructions.

2.2 Laboratory Studies

Physiological and molecular Responses of *An. gambiae* ss to cadmium, copper and lead were assessed.

2.2.1 Bioassays

Third instar *An. gambiae s.s* larvae were selected for tolerance to cadmium, copper and lead heavy metals concentrations (ppm).

2.2.1.1 Sources of Heavy Metals

Cadmium, copper and lead were evaluated as cadmium chloride ($CdCl_2$) 99.99% pure, copper II nitrate hydrate ($Cu(NO_3)_2$ $2.5H_2O$) >99 % pure and lead II nitrate ($Pb(NO_3)_2$) 99.5% pure analytical salts, sourced from Fisher Scientific, Fair Lawn, NJ, Sigma-Aldrich, Laborchemikalien, GMBH, Germany, and Prolabo, Fontenay, France respectively.

2.2.1.2 Test Insects

Mosquito test populations colony of *An. gambiae sensu stricto* were received from the Human Health Division of the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi Kenya. This colony was originally collected from Mbita field station (00025'S, 34013'E), South Nyanza province, Kenya in December, 2000 where *An. gambiae s.s* is abundant. At the time of this work, the colony was in the 35th filial generation post field sampling, away from any possible selection pressure by heavy metals.

2.2.1.3 Mosquito Rearing

The standard procedure for rearing *Anopheles* mosquitoes was followed. All life stages were reared in insectary (28 ± 2°C, 75 – 80 % Relative Humidity and 12L:12D photoperiod) of Animal and Rearing and Quarantine Unit (ARQU) of ICIPE, Nairobi, Kenya. From the day of emergence, adult mosquitoes were provided with a 10% sucrose solution soaked in cotton wool. Three day old female mosquitoes were allowed to feed on anaesthetized mice. Approximately 2-3 days later, oviposition dishes were placed in the cage containing gravid females. The eggs were placed on water and were surrounded with floating wax paper which served to keep eggs from becoming stranded on the sides of the hatching tray. Approximately 30 mg pulverized Tetramin fish food (Tetra GmbH, Melle, Germany) per pan was sprinkled on the surface of the water twice daily (three times daily after reaching the third larval stage). Pupae were collected daily, and transferred to the small bowls containing clean water. The bowls were placed in cages for adult emergence.

2.2.1.4 Heavy Metal Toxicological Selection Assay and Sampling for Molecular Studies

A strain of *An. gambiae s.s* third intar larvae that had previously not been exposed to heavy metals was separately placed under selection pressure with cadmium, copper and lead at concentration that caused 30% mortality (LC_{30}). Selection with median lethal concentration (LC_{50}) was evaluated and discarded after the selection survivors failed to emerge as adults, hence necessitating reduction in selection pressure. LC_{30} dosage was used to select *An. gambiae s.s* third instar larvae for F_1-F_5 generations. Survivals were raised as indicated above in the insectary in ARQU. Nine hundred larvae were selected for each generation and metal in three replicates each consisting of 300 larvae in 1500 ml water in polypropylene cylindrical pans with radius and height of 10.5 and 24.14 cm respectively. The susceptibility level of *An. gambiae* to each heavy metal in successive generations was monitored by determining the LC_{30} values as indicated below (2.2.4.1). A control colony was reared simultaneously and handled in the same manner through all manipulations but was not exposed to any heavy metals. The larvae were not fed during the 24 h exposure. Survivors were normally propagated. Fifty and 25 larvae were separately randomly selected from each of 24 h post exposure survivor replicates (each metal and control), placed in eppendorf tubes and immediately frozen at -70°C for subsequent molecular semi quantitative and two dimensional gel gene expression studies respectively. The remaining survivors were normally propagated.

2.2.1.4.1 Physiological Resistance Diagnostic Test

Toxicity range (24 h) of cadmium, copper and lead were conducted on each generation using third instar *An. gambiae* larvae. After determining the upper and lower toxicity ranges of each metals, a 24 h acute toxicity test were conducted. Three replicates (n = 25 per replicate) were exposed to five logarithmically separated lead, cadmium or copper concentrations within the established toxicity response range, in 400 ml of distilled water in the polypropylene cylindrical pans. Larval mortality was evaluated 24 h post exposure and LC_{30} determined by probit analysis as indicated below.

2.2.1.5 Effect of Heavy Metal Selections on Egg Hatchability, Larvae and Pupae Survivorship

Cohorts of 300 eggs were collected separately from fifth generation selection survivors of each heavy metal exposure and non-exposed control in three replicates. The eggs were placed in 1500 ml chlorine free distilled water and the egg hatchability examined microscopically 48 h post exposure. All resultant larvae after eclosion were counted and were propagated in 1500 ml of water. The numbers of larvae successfully pupating each day were counted into jars for emergence, kept separately by pan and date. The number of emerging adults male and female from each pan was recorded daily. The rearing was conducted in accordance with the standard procedure above (2.2.1.3).

2.2.1.6 Effect of Heavy Metal Selections on Adult Survivorship

The initial emergent females were immediately randomly sampled from each treatment for wing measurement (Briegel, 1990). A total of 29, 23, 32 and 19 females were

available for wing measurements following the selection from non-exposed control, cadmium, copper and lead selected populations respectively and the right wing consistently selected and measured. Briefly, Leica WILD M3Z dissecting microscope, was calibrated using a 2 mm microscope slide scale divided into 100 parts at 0.01 mm intervals, and a graduated eye-piece graticule. Under each magnification, the graduations on the microscope slide were matched with those on the eye-piece graticule. Perfectly coinciding graduation marks on the two scales were noted and the conversion factor calculated. The conversion factors established were 0.07692, 0.05128, 0.03226, 0.02083 and 0.0129 for 6.5, 10, 16, 25 and 40 times magnification respectively.

Subsequent emergent adults were collected for adult life studies by methods of Reisen and Mahmood (1980). Briefly, cohorts of 30 males and 30 females (less than 12 h old) from non-selected control and each heavy metal selected population were counted into separate plastic containers (4L) in three replicates. They were continuously offered 10% sucrose diet with cotton wool, and anaesthetized mice provided daily as blood meal source, and for oviposition, water in a plastic cup lined with filter paper (9 cm radius). Egg cups and sucrose cotton wool were changed daily. Males were provided with the 10% sucrose solution only. Mortality of females was daily recorded until when the last mosquito died. All experiments were conducted under standard insectary conditions above (2.2.1.3).

2.2.2 Molecular Responses of *An. gambiae s.s* to Selection by Cadmium, Copper and Lead Heavy Metals

Gene expression and proteomic profiles of *An. gambiae s.s* selection by heavy metals was conducted by looking at expression profiles of specific metal responsive genes and as differential expression of proteins respectively to heavy metals selection.

2.2.2.1 Isolation of Genomic DNA

Genomic DNA was extracted from 25 third instar *An. gambiae* larvae by phenol-chloroform DNA extraction method of Sambrook *et al.* (1989) with RNAase A treatment. Briefly, the larvae were homogenized in 120 µl DNA extraction buffer (0.4 M NaCl, 10mM Tris-HCl pH 8.0 and 2mM EDTA, pH 8.0) in 1.5 ml eppendorf with a hand-held motor with a polypropylene pellet pestle. 1.5 µl RNAse A was added and was further homogenized for another 30 seconds followed by incubation at 37°C for 60 minutes. Three microliters of Proteinase K (20µg/µl) was then added followed by further incubation at 50°C for 60 min. 121.2 µl of phenol: chloroform: isoamylalcohol (50:50:1) was added and microcentrifuged for 20 min at 14000 rpm at 4°C and room temperature and the top aqueous phase containing the DNA transferred to a new tube. 0.1 volumes of 2M sodium acetate pH 5.6 and 2.5 volumes of ice-cold absolute (100%) ethanol were added and gently inverted to mix. DNA was precipitated overnight at –20°C and microcentrifuged for 20 min at 14000 rpm at 4°C. The supernatant was discarded and the DNA pellet washed twice with 1 ml of 70% ethanol at 4°C. The DNA was microcentrifuged at 14000 rpm at 4°C for 20 min and the supernatant discarded after each wash. The pellet was air dried in a hood at room temperature and was subsequently

dissolved in 50-ul TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0) buffer, and quantified spectroscopically at 260 nm

2.2.2.2 Semi Quantitative RT-PCR Analysis of Metallothionein and Alpha Tubulin Gene Responses in *An. gambiae s.s* to Selection by Cadmium, Copper and Lead Heavy Metals

Gene expression profile was evaluated through analysis of expression profiles of metallothionein and alpha tubulin heavy metals responsive genes between heavy metals sensitive (control) and intolerant (resistant) strains.

2.2.2.2.1 Isolation of RNA

Total RNA were isolated from frozen samples, collected during selection above, through guanidine isothiocyanate based modified protocol of RNAgents Total RNA Isolation system (Promega, Madison, WI), according to the manufacturer's specifications. The pellet containing RNA was re-suspended in denaturing solution, precipitated at –20°C in isopropanol overnight and pelleted by centrifugation(14000 rpm, 4^0C, 20 min). The RNA was washed once using ice-cold 75 % ethanol and was resuspended in 50 µl nuclease free water. The RNA integrity was also checked by 1.0% agarose (Sigma – Aldrich Chemie, Gmbh) RNA denaturing gel electrophoresis in 1.4 % sodium phosphate, stained with 1 ug/ml ethidium bromide. All RNA isolates had approximately 2:1 28S:18S eukaryotic ribosomal RNAs quantities, suggesting that the isolates had not been degraded. The RNA samples were resuspended in 50 µl dionised-distillled water. RNA yield and qualities were spectrophotometrically determined at 230, 260 and 280 nm. All RNA isolates had

an OD_{260}:OD_{280} and OD_{260}:OD_{230} between 1.8 and 2.0 and 1.5 –2.0 respectively, indicating clean RNA isolates (Sambrook *et al.*, 1989).

2.2.2.2.2 Complimentary DNA (cDNA) Synthesis

Reverse transcription was conducted using TaqMan Reverse trancriptase reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, Oligo d $(T)_{16}$ was used as primer in the first step of cDNA synthesis in a reaction mix consisting of 1X TaqMan RT buffer, 5.5 mM $MgCl_2$, 1.5 µg Total RNA , 2.5 µM oligo-dT, 0.4 U/µl RNAse inhibitor, 500 µM dNTPs , 125 U/µl Multiscribe Reverse Transcriptase and H_20 in a total volume of 10 µl. The mix was incubated at 25°C for 10 min to maximize primer-RNA template binding, reverse transcribed at 48°C for 30 min and the reverse trancriptase inactivated at 95°C for 5 min. The cDNA stock was stored at -20°C until when required.

2.2.2.2.3 Selection and Design of Metal Responsive Gene Primers

Primers were designed from heavy metal responsive metallothionein (MTn A) from *Drosophila melanogaster* (Maroni *et al.*, 1987) and alpha tubulin from *C. tentans* (Mattingly *et al.*, 2001). Primers for ribosomal protein S7 (RP S7) were used as internal neutral/loading control gene. Metallothionein (Genebank accession # NM_079575) and alpha tubulin (Genebank accession # M14643) protein sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The functional homologues of alpha tubulin and metallothionein protein sequences in *An. gambiae* were determined by BLAST (Altschul *et al.*, 1997) analysis of the sequences

against *An. gambiae* genome and presence of domains and motifs in both genes were identified through InterProScan (Mulder *et al.,* 2003) analysis at the European Bioinformatics Institute (http://www.ebi.ac.uk/InterProScan). *An. gambiae* metalothionenin (Genebank accession # AAX86006) was isolated by the *Drosophila* MTn A holomolgy search. Alpha tubulin primer pairs were designed using primer3 software (Rozen and Skaletsky, 2000). Metallothionein primers were manually designed from protein sequence in such a way that the 5' and the 3' primers span different exons, so that the amplification product obtained from the cDNA was of different length from that obtained from any contaminant genomic (pseudogenes) DNA comprising intronic sequences. The protein sequences were back-translated with *An gambiae* codon bias into DNA sequences, using accessible Markus Fischer, Backtranslation Tool (http://www.entelechon.com/index.php?id=tools/backtranslation&lang=eng,Accession date July 20, 2003) with *An. gambiae* codon usage table (Entelechon GmbH, Regensburg, Germany). Primers generated were selected manually according to the following criteria: C+G content, >60 %; repetitive sequences, absent; repetitive bases, stretches of >3 identical bases (such as poly Ts) were avoided; sequence were selected perfectly homologous to target cDNA of interest and so that the 3' end base was preferably G or C. Additionally, alpha tubulin and metallothionein primers were chosen that were between 18 and 25 bases long, with Tm range within that of RP S7 and that would generate amplification products that would not overlap with that of RP S7 amplification product.

The following primers were subsequently selected: Metallothionein 5'- ATG CCC TGC AAG TGC TGT GG -3' (Tm 65.5 ^{o}C), and 5'- CTT GCC GCA GCA ACC GCC GGA

C-3' (72 °C) (annealing 55 °C) Alpha Tubulin , 5'- GAG CCG TAC AAC TCC ATC CTG -3' (62.1°C) and, 5'- GTA CGG CAC CAG ATT GGT CT -3' (61.5 °C) (annealing 53 °C) and RP S7 5'- GGC GAT CAT CAT CTA CGT TGC -3' (61.1°C) and, 5'- GTA GCT GCT GCA AAC TTC GG -3' (60.3°C) Anealing (53 °C). The melting (Tms) and annealing temperatures indicated in brackets were determined by using pDRAW32 software version 1.0 (ACA Clone Software). Expected primer specificity and sizes of genomic and cDNA amplification products were confirmed by alignment of the primers against their respective gDNA and cDNA sequences using Multalin program (Corpet, 1988). Metallothionein, Alpha Tubulin and RP S7 were expected to yield amplification products of 135, 199 and 497 bp according to this scheme. Metallothionein was also accordingly expected to yield amplification product of 213 bp on genomic DNA and was used to screen all cDNA synthesized for presence of genomic DNA carryover/contamination.

2.2.2.2.4 Screening of cDNA Products with Metallothionein primers for Genomic DNA Contamination

Standard PCR (2.2.2.2.8) was conducted on each individual cDNA sample and gDNA control. Both cDNA and gDNA products were co-electrophoresed and documented as indicated below (2.2.2.2.8). Any sample with genomic DNA contamination/carryover was re-extracted.

2.2.2.2.5 Optimization of Primer and $MgCl_2$ Concentration and Cycling Parameters

Primer and $MgCl_2$ concentrations and cycling parameter were optimized by methods of Morone *et al.,* (2001). Individual primers and $MgCl_2$ were evaluated in 0.05-1.00µg/µl and 2.50-7.5 mM concentration ranges respectively to choose the condition that gave the highest yield and specificity. 21-45 cycles were evaluated on the optimum primer $MgCl_2$, where 45 was considered the plateau stage control with the rest applied to determine appropriate numbers of cycles for production of quantifiable, clearly visible product on agarose gel, within amplification exponential range and could all be measured on the same gel. All optimization was conducted with none exposed control cDNA. Standard PCR reaction conditions (2.2.2.2.8) were applied with respective $MgCl_2$ concentrations and cycles adjustments. The PCR products were electrophoresed and gel documented as indicated below.

2.2.2.2.6 Control for Competition between Primer Sets

Competition between internal control RP S7 and either alpha tubulin, or metallothionein primer sets was eliminated by methods of Morone *et al.,* (2001). Control cDNA was separately amplified with alpha tubulin, metallothionein and internal control RP S7 primer sets. Additionally, same cDNA was amplified with RP S7 internal control at the same time in the presence of alpha tubulin and metallothionein primer sets separately. All the amplification products were run on the same agarose gel for quantitation to detect competition. Standard PCR reaction conditions below were applied but with optimized primer and $MgCl_2$ concentrations and cycles. The PCR products were electrophoresed

and gel documented as indicated below. When competition was detected, different reaction conditions were tested

2.2.2.2.7 Definitive Semi-Quantitative RT-PCR

The optimization process above established that 0.1 μg each of the primers, 5.5 mM $MgCl_2$ were optimum conditions for all primer sets for both metallothionein and alpha tubulin. Optimim cycles for metallothionein and alpha tubulin were also established as 22 and 25 respectively when multiplexed with RP S7 internal control. These conditions were subsequently applied on semi-quantitative RT-PCR on each cDNA sample synthesized above from RNA samples with metallothionein and alpha tubulin primer sets multiplexed with RP S7. The standard PCR conditions below, with adjustment to the optimum conditions, were applied. Gel electrophoresis and imaging was conducted as indicated below (2.2.2.2.8).

2.2.2.2.8 Standard PCR, Gel Electrophoresis and Imaging

Each PCR reactions consisted of 0.1μg of each primer set, 1μl cDNA/gDNA, 0.2 mM dNTP, 1 unit of Taq polymerase (Promega, Madison, MO) and 2 mM $MgCl_2$ in a total volume adjusted to 25 μl with H_2O. The thermo-cycling parameters were 94° C for 2 min followed by 45 cycles of 94° C for 1 min, 56° C for 1 min, 72° C for 1 min and a final extension at 72° C for 10 min. The PCR products were loaded onto Ethidium Bromide stained, 3 % agarose gels in TBE (Samrook et al, 1989). A 50 bp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run on every gel to confirm expected molecular weight of the amplification product. Gel images were acquired with a

Nikon CCD camera and quantification of the bands was performed by ImageQuant TL (Amersham Biosciences). Expression volumes/intensities were analysed and converted to cDNA nano grams (ng) using ImageQuant TL (Amersham Biosciences) 1D analysis software and calibration of the bands with 500 bp band of GeneRuler^{TM} 50 bp DNA ladder (Fermatas, Hanover, MD) according to the manufacturer's instructions.

2.2.2.3 Two-Dimensional Gel Electrophoresis Analysis of Differential Expression of Proteins in Third Instar *An. gambiae s.s* Larvae in Response to Selection by Cadmium, Copper and Lead Heavy Metals

Differentially induced protein spots in hird instar *An. gambiae ss* in response to cadmium, copper and lead were assessed.

2.2.2.3.1 Protein Extraction and Quantification

Protein was separately extracted from the triplicate samples, 50 larvae each, sampled and frozen (-70°C) above (2.2.1.4) from each generation and controls using phenol extraction followed by methanolic ammonium acetate precipitation methods of Hurkman and Tanaka (1986). Briefly, protein was separately extracted from triplicate samples, 50 larvae each sampled and frozen (- 70°C) above from each generation and controls using phenol extraction followed by methanolic ammonium acetate precipitation methods of Hurkman and Tanaka (1986) with modification. Briefly, 50 (approx 1g) mosquito larvae were ground to a powder with pestle, in liquid nitrogen in 1.5 ml eppendorf tube. 300 µl of Tris pH 8.8 buffered phenol and extraction media (0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9 M sucrose) each were immediately added and the

grinding continued for an additional 30 sec in a fume hood. The solution was further agitated by inversion for 30 min and then centrifuged for 10 min at 9000 rpm all at 4°C. The phenol phase was removed and the aqueous phase back-extracted with 300 µl each of the phenol and extraction media by vortexing. The resultant solution was centrifuged at 9000 rpm at 4°C as and the phenol phase was combined with first phenol extraction. Phenol extracted proteins were precipitated by adding 5 volumes of 0.1 M ammonium acetate in 100% methanol (stored at –20°C) to phenol phase all in 25 ml falcon tubes, followed by vortexing and incubating at –20°C overnight. The precipitate was collected by centrifugation for 20 min, at 14000 rpm at 4°C. The pellets were washed twice with 0.1 M ammonium acetate in methanol (stored at –20°C), twice with ice-cold 80% acetone and finally once with ice cold 70% ethanol. The pellet was completely resuspended each time with vortexing and gently pipeting. The resuspended sample was placed at –20°C for at least 15 min between each wash. The last suspended pellet was stored in 80% acetone at –20°C until isoelectric focusing was conducted. For protein quantitation, protein was acetone precipitated and the quantity determined by Bradford assay (Bradford, 1976).

2.2.2.3.2 Two-Dimensional Gel Electrophoresis and Imaging

Two dimensional polyacrilamide gel electrophoresis (2D-PAGE) was performed as described by O'Farrell, (1975). For each tube gel isoelectric focusing, the gel was pre-run for 15 min at 20 V, 30min at 30 V and 30 min at 40 V. 50 µg protein sample was loaded and isoelectric focused for 15 h at 40 V and 1 h at 80 V. Following isoelectric focusing, gels were equilibrated in 0.06 M Tris-HCl, pH 6.8, 2% SDS, 100 mM DTT and 10% glycerol. Separation in the second dimension was performed in 12 – 15% gradient

polyacrylamide (SDS-PAGE) gel. Proteins on analytical 2D-PAGE gels were visualized by coomersie blue staining and digitized with gel documentation system (Biosystematica, UK).

2.3 Data Analysis

Data obtained from the experiments above were analyzed as outlined below.

2.3.1 Analysis of the Impact of Heavy Metals and on Mosquito Species Composition and Spatial Distribution Patterns in Aquatic Larval Habitats in Urban Kisumu and Malindi

Comparisons of heavy metal prevalence in water and soil samples between towns, and among strata within each town were determined by one-way analysis of variance (ANOVA) with town and strata as factors, respectively. One-way ANOVA was also used to compare heavy metal prevalence between strata, and between natural and human-made potential larval habitats in the two cities and Tukey HSD post-hoc test was used to determine which strata were significantly different from each other in terms of mean values where the variance was significant. Student t test was used to compare heavy metal prevalence between Kisumu and Malindi, and between natural and man-made habitats.

Natural habitats were defined as any water bodies that did not originate from human activities while the reverse was the case with the human-made habitats. Natural habitats therefore included ponds, swamps, and springs. Human-made habitats included, but were

not limited to, domestic and industrial drainage ditches, discarded car tyres, sewage pools and water tanks. The above analyses were conducted on data of heavy metal concentrations in both surface water and bottom sediment. Pearson Correlation analysis was used to evaluate the relationship between heavy metal prevalence in the surface water and bottom sediments. The same analysis was used to determine the relationship between different heavy metal ions. Since some response variants had less than five entries, Fisher's Exact Test was conducted to establish the relationship between heavy metals concentrations in both surface water, and presence of *An. funestus, An. gambiae*, culicines of *Ae. aegypti* mosquito larvae in the habitats. For Fisher's Exact Test, concentrations of each of the heavy metals were dichotomized into low and high categories, relative to the mean concentrations, and the presence of mosquito species in each habitat dichotomized as either present or absent.

The Fisher's Exact Test was separately conducted on surface water and in bottom sediments of larval habitats in urban Kisumu and Malindi. Forty and 55 aqueous larval habitats and 21 and 20 bottom sediments were sampled in Kisumu and Malindi respectively. Independent variables were high or low heavy metal concentration categories. Dependent variables were presence or absence of the mosquito species. In all analyses, p values below 0.05 were considered significant.

2.3.2 Selection for Cadmium, Copper, and Lead Heavy Metal Resistance in *An. gambiae s.s*

Acute mortality response was corrected by Abbott's formula (Busvine, 1971) and then transformed to Probits (Finney, 1971) for linear regression analyses and 30% lethal concentration (LC_{30}) determination. Data sets with more than 10% control mortality were not considered for analysis (Finney, 1971). Patterns of larvae mortalities, pupae emergence and mortality as well as male and female adult emergence between the treatments were conducted through cumulative frequency distributions. Median larval mortality (LM_{50}), pupation (P_{50}), pupae mortality (PM_{50}), male emergence (EM_{50}) and female (EF_{50}) emergence times were determined by Probit analysis (Finney, 1971).

Horizontal life table analytical methods were applied on both juvenile and adult stages of various selection categories of *An. gambiae*, since the cohorts were distinct and were followed through time. Briefly, apparent or stage specific mortality was expressed in logarithmic form as a k-value or killing power (Haldane 1949, Varley and Gradwell 1960) and

$$k = \log_{10}(N_x/N_{x+1}) \qquad \text{(I)}$$

where N_x was the number entering stage x on which the mortality acts, and N_{x+1} was the number entering the following stage. The stages considered were egg (k_e), larvae (k_l), pupae(k_p) and adult.

For adult life tables characteristics, calculations procedures, formulae and rationale employed essentially followed Reisen and Mahmood (1980) and Elkinton, (1993). Age – specific survivorship (l_x) was determined as

$$l_x = y_x/y_0 \qquad \textbf{(II)}$$

where y_x = the number of males and females alive on each day x.

Type of adult survivorship was established through survivorship curves in which mean numbers surviving in each age category in non-selected control and each heavy metal selected populations were plotted on a log scale against age. Survivorships were categorized as type I if the rate of mortality increased at old age, type II if the relationship was linear, representing exponential decline in numbers and type III if the greatest rate of mortality occurs in the youngest age category.

Age-specific life expectancy (e_x) was computed as

$$e_x = T_x/l_x \qquad \textbf{(III)}$$

where

$$T_x = \sum_{x}^{w} L_x \qquad \textbf{(IV)}$$

and

$$L_x = (l_x + l_{x+1})/2 \qquad \textbf{(V)}$$

and w = the day the last individual died; ie e_1 = adult life at emergence in days.

In order to transmit *Plasmodium vivax, P. falciperum*, *P. malariae* or, the anopheline vector must survive for about 7, 8 and 14 days respectively, at similar temperature and humidity applied in this study, post blood feeding on infected host (Siddons, 1944). Therefore, assuming the infective meal is taken during the mosquitoes' 2nd and 3rd nights

of life, the potential infective portion of the population would consist of females not less than 10 days of age. Mean life expectancy at 10 days (e_{10}), was therefore computed for the control and heavy metals selected populations.

The net reproductive rate per cohort, or the total number of living females produced per female (R_0), was established as

$$R_0 = a \sum_{x=1}^{w} l_x m_x \qquad \textbf{(VI)}$$

where a = the mean proportion of females that survived from egg through adult emergence, and

$$m_x = E_x p \qquad \textbf{(VII)}$$

where E_x was the mean number of larvae (i.e. hatched eggs) produced per female per age interval x, and p was the proportion of the offspring that are female. In this study, "a" for non-selected control, cadmium, copper and lead selected populations averaged 0.340, 0.096, 0.015 and 0.110 respectively and p were 0.56, 0.52, 0.50, and 0.51, respectively. The p values were based on observed sex ratio of the emerging adults from non-selected control and heavy metals selected populations.

Age of mean cohort reproduction in days (T_0) was established as

$$T_0 = a \sum_{x}^{w} l_x m_x x / R_0 \qquad \textbf{(VIII)}$$

starting at x =1, the day of adult emergence.

The instantaneous rate of increase in females per female (r_m), was calculated using the Dobzhansky *et al.*, (1964) modification of the original Euler-Lotka equation by the Newton Raphason iteration method where

$$1 = a\sum_{x=1}^{w} l_x m_x e^{-r_m(x+D)} \quad \textbf{(IX)}$$

where e is the base of natural logarithm and D is the length of time from oviposition in the present generation to first oviposition in the offspring generation. D was currently considered to be the observed mean median emergence time for females plus the duration of nuliperous period for that cohort. For non-selected control, cadmium, copper and lead selected populations, D ranged were 16.5 –17.50, 17.0 –19.0, 16.5 – 19.0 and 14.0 – 14.0 days respectively.

Mean generation time in days (G) was computed as

$$G = \ln R_0/r_m. \quad \textbf{(X)}$$

Since this value included D in its calculation, G was a realistic estimate of the time from mean oviposition in the present generation to mean oviposition on the offspring generation.

Instantaneous birth rate (b) was calculated as

$$b = \ln (1+\beta) \quad \textbf{(XI)}$$

and instantaneous death rate (d)as

$$d = (b-r_m) \quad \textbf{(XII)}$$

where

$$1/\beta = \sum_{x=1}^{w} L_x e^{-r_m(x+1)} \quad \textbf{(XIII)}$$

(Birch 1948)

Population doubling time (T_d) was calculated as

$$T_d = \frac{\ln(2)}{r} \quad \text{(XIV)}$$

(Elkinton, 1993)

Egg viability /hatchability, Mean larval mortality pupation, pupae mortality, male and female emergence, wing lengths, sex ratios, fecundity, k- values, male and female mean life expectancy from emergence (e_1), net reproductive rate, mean life expectancy at 10 days (e_{10}), (R_o) age at mean cohort reproduction (T_o), instantaneous rate of increase (r_m), mean generation time (G), instantaneous birth (b), death rate (d), population doubling time (T_d), r_m/b and b/d among the treatments were compared by one way ANOVA with control and heavy metals selected populations as factor. Means that were significantly different were separated by Tukey HSD post-hoc analysis.

2.3.3 Semi Quantitative RT-PCR Analysis of Metallothionein and Alpha Tubulin Gene Responses in *An. gambiae s.s* to Selection by Cadmium, Copper and Lead Heavy Metals

Band intensity was expressed as relative absorbance units with background subtracted. The ratio between Metallothionein/alpha tubulin and RP S7 amplicon volumes on each cDNA was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard error of mean of all experiments performed were calculated after normalization to RP S7. Additionally three ways interactive ANOVA was conducted on the normalized volumes with heavy metal type, generation and gene as factors.

2.3.4 Two-Dimensional Gel Electrophoresis Analysis of Differential Expression of Proteins in Third Instar *An. gambiae s.s* Larvae in Response to Selection by Cadmium, Copper and Lead Heavy Metals

Second dimension gels were analyzed by Phoretix software (Nonlinear Dynamics, Newcastle, UK). Normalized protein spots were detected (Appendix 6.5) and apparent molecular weights of the protein spots were determined by analysis against the co-electrophoresed 10–200 KDa SDS-PAGE molecular weight standards (Fermantas, Lithuania) (Appendix 6.6). Apparent isoelectric points (pI) of each protein spot was determined by calibration against the 3–10 pH gradient of IEF electrophoresis (Appendix 6.7). Differentially expressed protein spots from control and heavy metals selected population from respective generations were detected by overlying and the warping of similar spots to each other in the gels (Appendix 6.8).

Results obtained by the computer-aided evaluation were rigorously compared by visual analysis of the original gels. Changes in specific polypeptides were recorded only when they occurred in all the replicated gels. Quantitative comparison between gels was achieved using normalized spot volumes. Differences without or equal to ± 1.5-fold change in any of the treatments against control among matched protein spots were considered significant. The spots were therefore categorized as up, down regulated or unaffected if the differences were greater than or equal to 1.5, less than or equal to -1.5 or within ± <1.5 fold change respectively. If these differences were subsequently reproduced in additional experiments, despite possibly being of lower magnitude, they were held to be consistent. Both differentially and constitutively expressed protein spots

with pH less than, equal to or greater than seven were categorized as acidic, neutral or basic respectively. The spots were also categorized as having low, medium or high molecular weight if they were 0–69, 70-133 and 134–200 KDa, respectively.

Patterns of distributions of differentially against constitutively expressed protein spots were determined by Chi Square and Fishers exact test. Comparison of magnitude of differentially expressed spots, between and within generation, among metals and control were conducted by three-way ANOVA with metal/control, generation, and interaction between the two as factors. One-way ANOVA was separately conducted on each metal across generations where interaction was significant and the means separated by Tukey HSD post hoc test where the difference was significant. Variations in pH, molecular weight, and expression profiles folds between categories was determined by Chi Square. All statistics were conducted through SPSS (SPSS Corporation, Chicago, Illinois Statistical Package version 11.5).

Chapter 3

Results

3.1 Heavy Metals and their Impact on Mosquito Species Composition and Spatial Distribution Patterns in Aquatic Larval Habitats in Urban Kisumu and Malindi

There were variations in mosquito species composition in larval habitats in the urban centers. The distributions of heavy metals in urban larval habitats were also variable among surface water and sediment samples. There was correlation between some heavy metals and presence of some mosquito species.

3.1.1 Mosquito Species Composition in the Larval Habitats

Temporary, semi temporary and permanent habitats constituted 15.8, 61.4 and 22.8%, respectively, in Kisumu. The same categorie of habitats constituted 33.8, 28.6 and 37.7% respectively in Malindi. In Kisumu and Malindi 77 and 95 % of the habitats respectively, were man-made. Only 28.6 and 48.1% of the sampled habitats in Kisumu and Malindi, respectively, contained mosquito larvae. Out of the 93 mosquito larvae collected in Kisumu, only about 16% were anopheline (*An. gambiae,* 11% and *An. funestus,* 5%). Most of the *An. gambiae* collected are most likely *An. arabiensis* since previous results indicate that majority of An. *gambiae* in Kisumu are *An. arabiensis* (Githeko *et al.,* 1993). The rest were *Cx quinquefasciatus* say, 1823. In Malindi, 4% of all the collected larvae were *An. gambiae* and > 95% were *culicine spp (Ae. aegypti* and *Cx. quenquifasciatus*). No *An. funestus* was collected in any of the larval habitats sampled in Malindi. Most mosquitoes (71.2%) were collected from unplanned and poorly drained strata in Kisumu. The majority (86.57%) of these were *Cx.*

quenquifasciatus species, which were also dominant (90.1%) in the planned and well-drained strata. No mosquitoes were sampled in the unplanned and well-drained strata. Similarly, unplanned and poorly drained strata had the highest proportion (58.0%) of mosquitoes in Malindi. Most (98.3%) of these were *Ae. aegypti. Aedes aegypti* was also dominant (> 50.0%) in all the other strata. Planned and poorly drained strata had the least proportion (5.5 %) of mosquitoes. Planned and well-drained strata had 17.5% of the mosquito population among which were dominated (87.0%) by *Ae. aegypti*.

3.1.2 Distribution of Heavy Metals in Urban Larval Habitats

Heavy metal levels were determined in both surface water and sediments in larval habitats in various strata in urban Kisumu and Malindi

3.1.2.1 Surface Water

The most prevalent metal in larval habitats in Kisumu was manganese while this was the case with iron in Malindi. (Fig 1) Manganese was also more prevalent in Kisumu than in Malindi ($p < 0.05$). Cadmium was least prevalent metal in Kisumu and Malindi. Comparison of heavy metals concentrations between habitats in Kisumu and Malindi revealed similar ($p > 0.05$) concentrations of all ions except manganese. Copper, lead and zinc were higher in larval habitats in Malindi than in Kisumu. The concentrations of most of the metals were above the WHO permissible limit in drinking water (WHO, 1996) (Fig 1)

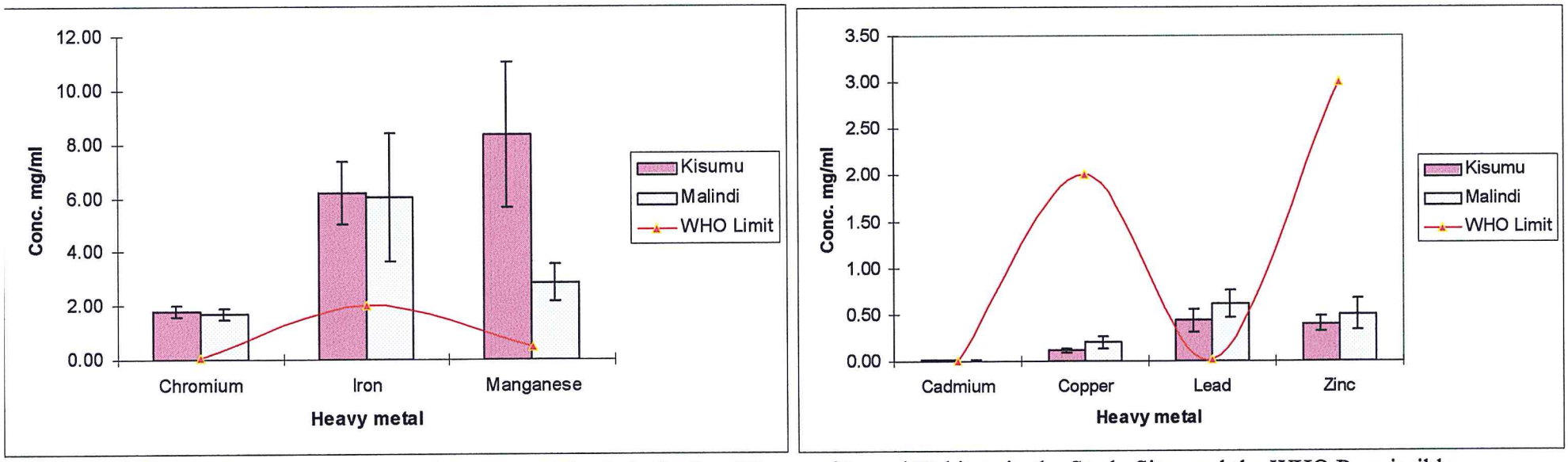

Fig 1: Concentrations of various Heavy Metals in Surface Waters of Larval Habitats in the Study Sites and the WHO Permissible level in Drinking Water

3.1.2.2 Sediments

Iron and cadmium were the most and least prevalent heavy metals, respectively, in the sediments of larval habitats in Kisumu and Malindi. The concentrations of iron were 767.6 ± 90.2 and 366.5 ± 84.8 mg/kg in larval habitats in Kisumu and Malindi, respectively. Concentrations of cadmium in larval habitats in Kisumu and Malindi were 0.009 ± 0.003 and 0.004 ± 0.002 mg/kg respectively. However, manganese and iron were more prevalent ($p>0.05$) in sediments of larval habitats in Kisumu than in Malindi. Concentrations of the rest of the metals were similar ($p<0.05$) between cities.

Among the heavy metals analyzed, only cadmium prevalence was significantly correlated ($p<0.05$) between the aqueous and sediments samples in larval habitats in Malindi. Similarly, only cadmium and copper prevalence were significantly correlated ($p<0.05$) between water and sediments in larval habitats in urban Kisumu. All the correlations were positive. More significant correlations among heavy metals were detected in Malindi as compared to Kisumu samples (Table 1). A similar trend was evident in the sediment samples (Table 2).

Table 1: Pearson Correlation coefficients (r) for correlations between various heavy metals in surface water of various larval habitats in urban Kisumu (n = 40) and Malindi (n = 55)

	Cadmium		Chromium		Copper		Iron		Lead		Manganese	
	Kisumu	**Malindi**	**Kisumu**	**Malindi**	**Kisumu**	**Malindi**	**Kisumu**	**Malindi**	**Kisumu**	**Malindi**	**Kisumu**	**Malindi**
Chromium	-0.08	0.14										
Copper	0.48**	0.33*	0.08	0.55**								
Iron	0.31	0.27*	0.08	0.34*	0.04	0.91**						
Lead	0.44**	0.19	-0.13	0.29*	0.82**	0.51**	0.00	0.45**				
Manganese	0.22	0.14	0.22	0.48**	0.52**	0.60**	0.31	0.46**	0.35*	0.26		
Zinc	0.11	0.34*	0.42**	0.45**	0.26	0.93**	0.46**	0.94**	0.05	0.46**	0.79**	0.56**

** Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

Table 2: Pearson Correlation coefficients (r) for correlations between various heavy metals in bottom sediments of various larval habitats in urban Kisumu (n = 21) and Malindi (n= 20)

	Cadmium		Chromium		Copper		Iron		Lead		Manganese	
	Kisumu	**Malindi**	**Kisumu**	**Malindi**	**Kisumu**	**Malindi**	**Kisumu**	**Malindi**	**Kisumu**	**Malindi**	**Kisumu**	**Malindi**
Chromium	-0.16	0.07										
Copper	0.39	-0.14	-0.06	0.06								
Iron	0.07	-0.07	0.38	0.28	0.24	0.72**						
Lead	-0.02	-0.13	0.03	0.15	0.15	0.92**	0.29	0.82**				
Manganese	0.18	0.15	0.4	0.3	0.35	-0.04	0.62**	0.45*	0.39	-0.03		
Zinc	-0.24	0.16	0.01	0.09	-0.13	0.51*	0.15	0.44	0.05	0.35	0.11	0.33

** Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

Distributions of any of the heavy metals in water of larval habitats in Kisumu among the strata were similar ($p>0.05$). There was however significant ($p<0.05$) variation in distribution of copper, iron and zinc in urban Malindi. Most of these metals were concentrated in unplanned, well-drained strata. The concentrations of copper in the strata were 0.076 ± 0.014 (well-planned well drained), 0.123 ± 0.049 (well planned-poorly drained), 1.041 ± 0.738 (poorly-planned well-drained) and 0.172 ± 0.050 (poorly-planned poorly drained) ppm while those of iron were 2.931 ± 0.479 (well-planned well drained), 3.242 ± 0.725 (well planned-poorly drained), 38.857 ± 31.682 (poorly-planned well-drained) and 3.794 ± 0.426 (poorly-planned poorly drained) ppm. Zinc concentrations were 0.245 ± 0.107 (well-planned well drained), 0.218 ± 0.083 (well planned-poorly drained), 3.206 ± 2.055 (poorly-planned well-drained) and 0.344 ± 0.088 (poorly-planned poorly drained) ppm. Few differences in distribution of each of the heavy metals were detected in sediments from larval habitats in Kisumu, suggesting relative homogeneity in distribution across strata and in agreement with the observation on the water sample. However, in Malindi, the distribution of zinc was heterogeneous among the strata while that of the rest of the metals was homogenous. The zinc concentrations were 1.089 ± 0.181, 0.941 ± 0.211, 2.575 ± 0.363 and 0.829 ± 0.304 ppm in well-planned well-drained, well-planned poorly-drained, poorly-planned and well-drained, and poorly-planned poorly-drained, respectively.

Concentrations of all the heavy metals were consistently higher in water from man-made than natural larval habitats in Kisumu and Malindi though the difference was not significant ($p>0.05$). Most of the heavy metal concentrations were higher in sediments from natural than in man-made larval habitats in Kisumu. Concentrations of each of the

heavy metals in the sediments in Malindi were similar ($p>0.05$). From the natural and man-made larval habitats findings were consistent with those in the surface water were made.

3.1.3 Heavy Metals and Mosquito Presence

A significant positive association was observed between copper concentration in the surface water and *Ae. aegypti* presence in Kisumu, Similarly, significant association was revealed between lead concentration in surface water and *An. gambiae* and *Ae. aegypti* presence ($p<0.05$) in Kisumu. There were no significant ($p>0.05$) associations between concentrations of the remaining heavy metals and mosquito presence in Kisumu. There were similarly no significant ($p>0.05$) associations between any of the heavy metal concentrations in the surface water or bottom sediments with presence of *An. funestus, An. gambiae, Ae. aegypti* and culicine mosquitoes in Malindi.

3.2 Effect of Cadmium, Copper and Lead on *An. gambiae ss* Biological fitness

The biological responses of *An. gambiae ss* to heavy metal selection was evaluated.

3.2.1 Toxicological Response

As indicated in Table 3, there were variations in toxicological responses of third instar *An. gambiae* to selection by heavy metals as indicated in Table 3. In that respect, LC_{30} was highest in response to lead compared to copper and cadmium selection; with cadmiun have the lowest LC_{30} across all generations. There was an overall 13, 11 and 78 folds increase in LC_{30} following selection by cadmium, copper and lead, respectively.

The most increase in magnitude of LC_{30} was observed between generations 2 and 3, 3 and 4, and 1 and 2 following cadmium, copper and lead selections, respectively. The least increase was between the fourth and fifth generation in all heavy metals categories.

Table 3: Responses (LC_{30}) of various generations of third instar *An. gambiae* larvae (n = 25, replicates = 3) to successive generational Selection by various heavy metals

Generation	Cadmium	Copper	Lead
1	0.47 (0.36 - 0.58)	1.04 (0.57 -1.30)	10.37 (5.56 - 12.99)
2	0.99 (0.20 - 1.81)	1.91 (1.08 - 4.45)	80.27 (27.36 - 146.39)
3	5.44 (2.47 - 7.76)	2.28 (0.63 - 4.31)	473.00 (96.96 - 1370.49)
4	5.10 (2.95 - 8.38)	9.14 (3.11 - 38.79)	791.31 (433.51 -1805.95)
5	6.15 (2.83 - 8.70)	11.15 (2.35 -18.33)	810.00 (680.51 - 2261.95)

Figures indicated in brackets are 95% confidence intervals about respective median value.

3.2.2 Developmental Attributes of Non-Selected Control and Heavy Metal Selected *An. gambiae ss* Populations

Developmental stages on *An. gambies* were influenced in various ways by heavy metals selections as illustrated in Table 4. Specifically, egg hatching was significantly ($p<0.05$) higher in the control than in any of the heavy metals selected populations. The hatching was however similar among the heavy metals selected populations. Larval mortality was significantly ($p<0.05$) lower, in the control than in each of the heavy metals selected populations. Among the heavy metals category, larvae mortality in cadmium-selected population was significantly ($p<0.05$) higher than that from copper selection, but both mortalities were similar ($p>0.05$) to those observed in lead selected populations.

The magnitude of pupation was significantly ($p<0.05$) higher in control than in any of the heavy metal selected populations. The magnitude was about three folds higher in control than in cadmium, and two-folds higher than in both copper and lead selected populations. Among the heavy metals, pupation was significantly ($p<0.05$) higher in copper than in cadmium-selected populations. Pupae mortality was however similar ($p>0.05$) among all heavy metals selected populations and control.

The sex ratio of emerging male and female population among the control and heavy metal selected populations were similar ($p>0.05$). However, more adults of both sexes significantly ($p<0.05$) emerged from the control than in each of the heavy metals selected populations. Among heavy metals selected populations similar ($p>0.05$) number of females emerged among the heavy metals selected populations. The emerging female adults from the non-selected control had longer wings than those from any of the heavy metals selected populations, though the lengths were statistically similar ($p>0.05$). Fecundity of control population was significantly ($p<0.05$) higher than that of each of the heavy metals selected populations. The fecundities were however similar ($p>0.05$) among the heavy metals selected populations. Significantly ($p<0.05$) more males emerged from copper than from either cadmium or lead selected population.

Table 4. Developmental attributes of non-selected control and heavy metals selected *An. gambiae* populations.

	Eggs		Larvae		Pupae			
	Exposed	Hatched	Mortality		Emergence		Mortality	
Selection	n*	Mean	n	Mean	n	Mean	n	Mean
Control	300	299 ± 0.58^{a}	282	94.00 ± 6.93^{a}	618	206.00 ± 6.93^{a}	71	23.67 ± 1.86^{a}
Cadmium	300	261 ± 1.76^{b}	654	218.00 ± 3.51^{b}	246	82.00 ± 3.51^{b}	81	27.00 ± 2.89^{a}
Copper	300	256 ± 2.33^{b}	561	187.00 ± 2.52^{c}	339	113.00 ± 2.52^{c}	69	23.00 ± 3.51^{a}
Lead	300	266 ± 2.31^{b}	613	204.33 ± 7.45^{bc}	287	95.67 ± 7.45^{bc}	93	29.67 ± 2.67^{a}

	Adults								
	Emergence				Sex ratio	Female			
	Male		Female		(male/total)	Wing length		Fecundity	
Selection	n	Mean	n	Mean	Mean	n	Mean	n	Mean
Control	241	80.33 ± 0.33^{a}	306	102.00 ± 5.29^{a}	0.441 ± 0.013^{a}	29	3.202 ± 0.035^{a}	7084	2361.33 ± 35.03^{a}
Cadmium	79	26.33 ± 2.03^{b}	86	28.67 ± 3.84^{b}	0.483 ± 0.041^{a}	23	3.173 ± 0.029^{a}	2990	996.67 ± 92.85^{b}
Copper	135	45.00 ± 4.04^{c}	135	45.00 ± 4.04^{b}	0.500 ± 0.044^{a}	32	3.109 ± 0.033^{a}	3777	1134.00 ± 108.67^{b}
Lead	95	31.67 ± 3.48^{b}	99	33.00 ± 6.43^{b}	0.496 ± 0.039^{a}	19	3.175 ± 0.040^{a}	2709	1259.00 ± 101.89^{b}

Mean= mean ± Standard error of the mean (SEM) Different letters (superscripts) denote mean differences that are significant at the 0.05 level by Tukey HSD multiple comparisons in the column. * indicate number per replicate while All the other values (n) indicate the total number from the three replicates.

Various magnitudes of killing powers were operational at the specific juvenile stages of *An. gambiae* as indicated in Table 5. Specifically, egg-killing power (k_e) was similar ($p>0.05$) among the heavy metal selected populations, but was significantly ($p<0.05$) higher than in the control population. Copper had the highest k_e while lead had the least. Larvae killing power (k_l) was significantly ($p<0.05$) higher in each of the heavy metals selected populations than in the control. The power was five folds higher in copper and four folds higher in both copper and lead selected populations than in the control populations. The k_l in cadmium-selected population was higher than in copper and lead selected populations, though the magnitude was not statistically ($p>0.05$) different. Pupae killing power (k_p) was significantly ($p<0.05$) lower in control populations than in all but lead selected populations. Among the heavy metals, cadmium selected population had higher k_p, than both copper and lead selected populations. Overall, the highest stage specific killing occurred at larval stage and in cadmium selected population. The least affected stage was the larvae and in control population.

Table 5: Mean (± SEM) stage specific killing power (*k*-value) in non-selected control and various heavy metals selected populations

Stage	Control	Cadmium	Copper	Lead
Eggs	0.00 ± 0.00^{a}	0.06 ± 0.01^{b}	0.07 ± 0.01^{b}	0.05 ± 0.01^{b}
Larvae	0.16 ± 0.01^{a}	0.79 ± 0.06^{b}	0.58 ± 0.05^{b}	0.66 ± 0.08^{b}
Pupae	0.05 ± 0.00^{a}	0.46 ± 0.08^{b}	0.17 ± 0.01^{ab}	0.36 ± 0.11^{a}
Total	0.22 ± 0.01^{a}	1.32 ± 0.14^{b}	0.82 ± 0.06^{b}	1.06 ± 0.19^{b}

Different letters (superscripts) denote mean differences that are significant at the 0.05 level by Tukey HSD multiple comparisons within the row

The developmental changes occurred at various rates in heavy metals selected as control populations as shown in Table 6. Specifically, larval mortality, at median larvae mortality time (LM_{50}), was slower in control than in any of the heavy metals selected populations. It took three, seven and six more days to achieve LM_{50} in cadmium, copper and lead selected populations, respectively, than in control. Among the heavy metals, it took longer to achieve LM_{50} in cadmium than in either copper or lead selected populations. The rate of pupation, at the median pupation time (P_{50}), was delayed by about two days in cadmium and copper selected, relative to control populations, though the delay was absent in lead selected populations. The rate of pupae mortality, considered at median pupae mortality time (PM_{50}), was considerably slower in cadmium and copper selected than in control and lead selected populations. The (PM_{50}) occurred about four and two days later in cadmium and copper selected than in control populations. The male and female emergence at, median male (EM_{50}) and female (EF_{50}) emergence times were delayed by about two days in cadmium and copper selected populations, relative to control population, while there was no delay in the lead selected populations

Table 6. Median larval mortality, pupation, pupae mortality, male and female emergence times (days) of non selected control and heavy metals selected *An. gambiae.*

	Control		Cadmium		Copper		Lead	
Median	n	Days	n	Days	n	Days	n	Days
LM_{50}	282	12.97 (12.10 - 13.91)	654	9.70 (8.57 - 10.88)	561	6.41 (5.79 - 6.98)	613	7.24 (6.65 - 7.80)
P_{50}	618	13.25 (12.96 - 13.53)	246	15.59 (15.29 - 15.90)	339	15.09 (14.85 - 15.33)	287	13.20 (12.96 - 13.44)
PM_{50}	71	13.43 (12.77 - 14.09)	81	16.93 (16.41 - 17.49)	69	15.71 (15.11 - 16.31)	93	13.63 (13.16 - 14.09)
EM_{50}	241	13.19 (12.87 - 13.50)	79	14.82 (14.15 - 15.40)	135	14.97 (14.56 - 15.37)	95	12.87 (12.39 - 13.32)
EF_{50}	306	13.26 (12.98 - 13.54)	86	14.86 (14.27 - 15.38)	135	14.87 (14.50 - 15.24)	99	12.86 (12.42 - 13.31)

LM_{50}, P_{50}, PM_{50}, EM_{50}, EF_{50} are median larval mortality, pupation, pupae mortality, male and female emergence times respectively. Figures indicated in brackets are 95% confidence intervals about respective median value.

The rates of both male and female adult mortalities were similar among heavy metals selected and control populations in the initial 15 days of survivorship, and then rapidly increasing in the heavy metal selected than in the control populations in old age (Fig. 2). Among the heavy metals, the greatest relative increase occurred in the cadmium-selected populations (Fig. 2)

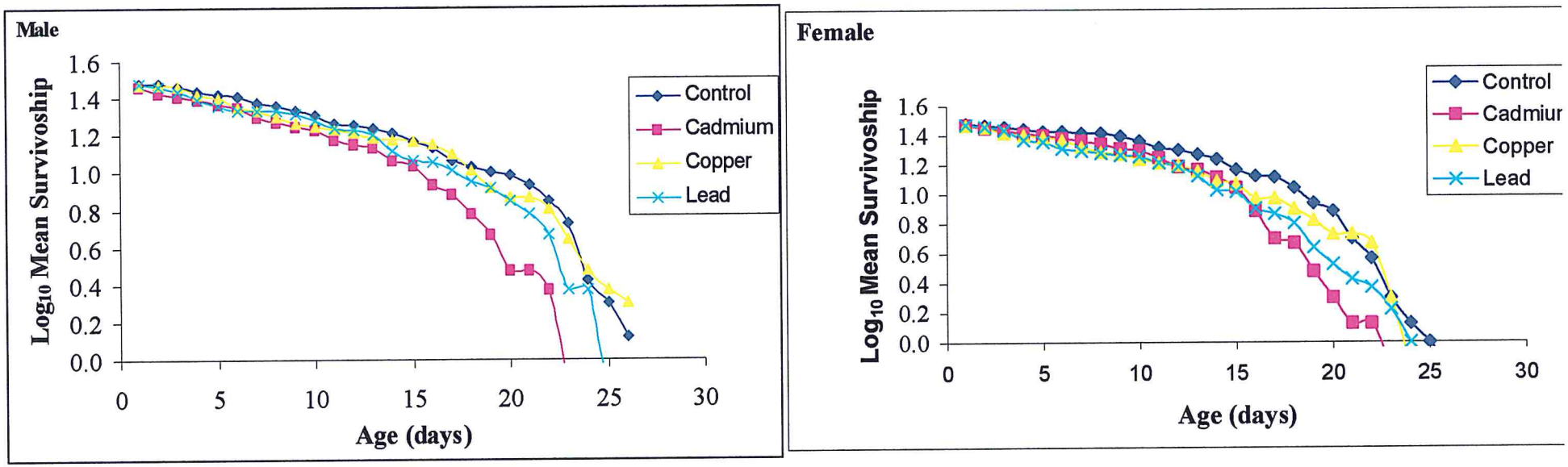

Fig. 2. Survivorship (log_{10} mean) curves for male and female non-selected control and various heavy metal selected *An. gambiae* adult

There were various differences in horizontal life table attributes between and among various heavy metals selected and control populations as indicated in Table 7. Specifically, there were no significant ($p>0.05$) differences in both male and female life expectancy (e_1) at emergence among control and heavy metal selected populations. Among the heavy metals, copper selected populations had higher e_1 than either cadmium or lead selected populations. Mean life expectancies at 10 days (e_{10}) were similar ($p>0.05$) between control and heavy metals selected populations. The net reproductive effort (R_o) of control was significantly ($p<0.05$) higher than that of each of the heavy metal selected population and were 12, 18 and 10 folds higher in cadmium, copper and lead selected populations, respectively. The R_o was however statistically similar ($p>0.05$) among the heavy metals selected populations. Ages at means cohort reproduction (T_o) among control and heavy metals selected populations were similar ($p>0.05$). However, the innate rate of increase (r_m) and mean generation time (G) were significantly ($p<0.05$) higher in the control than in the heavy metal selected populations, among which both parameters were similar ($p<0.05$). Instantaneous birth rate (b) was significantly higher in the control than in all, but lead selected populations. The b was however similar ($p>0.05$) among heavy metals selected populations. Rm/b as well as b/d ratios among the non-selected and heavy metal selected populations were similar ($p>0.05$), while the population doubling time (T_d) was significantly ($p<0.05$) longer in the heavy metals selected than in control populations.

Table 7. Adult life table characteristics of non- selected control and various heavy metals selected *An. gambiae* populations

Attribute		Control	Cadmium	Copper	Lead
e_1	male	7.35 ± 0.52^a	6.27 ± 0.19^a	7.36 ± 0.72^a	6.84 ± 0.38^a
	female	7.01 ± 0.32^a	5.81 ± 0.52^a	6.70 ± 0.28^a	5.65 ± 0.84^a
e_{10}		4.02 ± 0.29^a	3.04 ± 0.46^a	4.29 ± 0.47^a	3.39 ± 0.79^a
R_o		350.21 ± 13.00^a	29.07 ± 1.33^b	43.38 ± 8.77^b	34.52 ± 10.88^b
T_o		7.68 ± 0.52^a	7.57 ± 0.41^a	8.43 ± 0.17^a	9.19 ± 0.69^a
r_m		0.30 ± 0.01^a	0.23 ± 0.00^b	0.23 ± 0.01^b	0.23 ± 0.01^b
G		19.57 ± 0.44^a	14.80 ± 0.32^b	16.14 ± 0.31^b	14.77 ± 0.91^b
b		0.32 ± 0.01^a	0.26 ± 0.01^b	0.27 ± 0.00^b	0.28 ± 0.01^{ab}
d		0.02 ± 0.00^a	0.04 ± 0.01^a	0.04 ± 0.01^a	0.05 ± 0.02^a
T_d		2.32 ± 0.06^a	3.05 ± 0.06^b	3.02 ± 0.15^b	3.01 ± 0.14^b
r_m/b		0.94 ± 0.01^a	0.86 ± 0.02^a	0.84 ± 0.04^a	0.83 ± 0.08^a
b/d		20.25 ± 4.91^a	7.44 ± 0.96^a	7.18 ± 1.51^a	9.43 ± 4.32^a

Different letters (superscripts) denote mean differences that are significant at the 0.05 level by Tukey HSD multiple comparisons in the row. The experiments were conducted in three replicates, each consisting of cohorts of 30 males and 30 females, separately. e1 = mean life expectancy from emergence in days; e_{10} = mean life expectancy at 10 days post emergence. R_o= net reproductive rate in living female progeny per female per generation; T_o =age in days at mean cohort reproduction; r_m = instantaneous rate of increase in living female per female; G = mean generation time in days; b = instantaneous birth; d = death rate, assuming stable age distribution and T_d = population doubling time.

3.3 Molecular Responses of *An. gambiae s.s* to Selection by Cadmium, Copper and Lead Heavy Metals

Semi Quantitative RT-PCR analysis revealed differential induction of some specific heavy metals responsive genes in third instar *An. gambiae s.s* larvae in response to selection by heavy metals through various generations. Two dimensional gel electrophoresis analysis similarly identified differential expression of proteins in third instar *An. gambiae s.s* larvae, relative to the control.

3.3.1 Semi Quantitative RT-PCR Analysis of Metallothionein and Alpha Tubulin Gene Responses in *An. gambiae s.s* to Selection by Cadmium, Copper and Lead Heavy Metals

There were significant variations in expressions of metallothionein and alpha tubulin heavy metals responsive genes to selection by cadmium, copper and lead heavy metals in third instar *An. gambiae* larvae. None of the cDNA amplification products of metalthionein screening of each cDNA sample had additional genomic DNA equivalent amplification product (Fig 3) indicating that RNA extract from each sample was essentially free of genomic DNA.

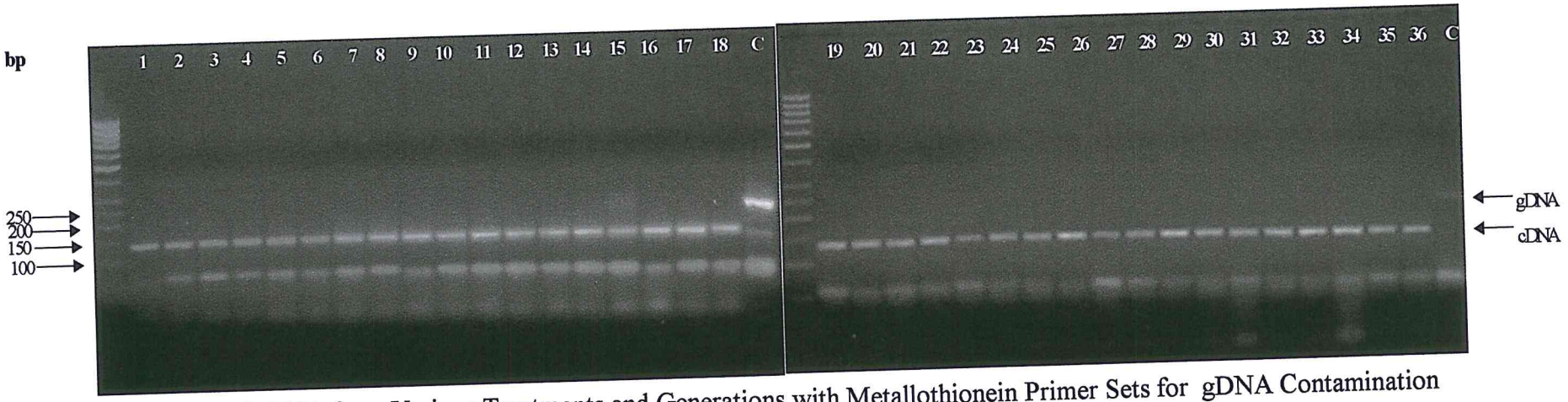

Fig 3: Screening of cDNA from Various Treatments and Generations with Metallothionein Primer Sets for gDNA Contamination

Samples from first, third and fifth generations were run in Lanes 1-12, 13-24 and 25-36 respectively. Among the lanes, 1-3, 13-15 and 2
27 are control. Lanes 4-6, 16-18 and 28-30 are from cadmium selected population, Lanes 7-9, 19-21and 31-33 from copper select
population and lanes 10-12, 22-24, and 34-36 from lead selected populations. Lanes with amplified genomic control DNA are indicat
with 'C'.

3.3.1.1 Control for Competition between Primer Sets

Neither Metallothionein nor alpha tubulin primers sets competed with RP S7 in the selected conditions of 5.5 mM $MgCl_2$, 56°C for both genes and 22 and 25 cycles for metallothionein and alpha tubulin respectively. There was similarity in both intensity and expression volumes between alpha tubulin/metallothionein alone and when multiplexed with RSP 7 (Fig 2). The expression volumes for RP S7 (314.908 ± 62.684) alone and when multiplexed with metallothionein (237.685 ± 62.597) were similar, so were those of metallothionein alone (181.764 ± 64.037) and in RP S7 multiplex (216.012± 61.613). RP S7 volumes alone (347.397 ± 66.526), and when multiplexed with alpha tubulin (395.452± 73.374), were also similar, and so were those of alpha tubulin alone (153.903± 72.967) and when multiplexed with RP S7 (157.905± 72.650). The volumes indicated are nano-gram (ng) of cDNA amplicons ± standard error of the mean (SEM). The conditions were subsequently adopted as definitive test parameters.

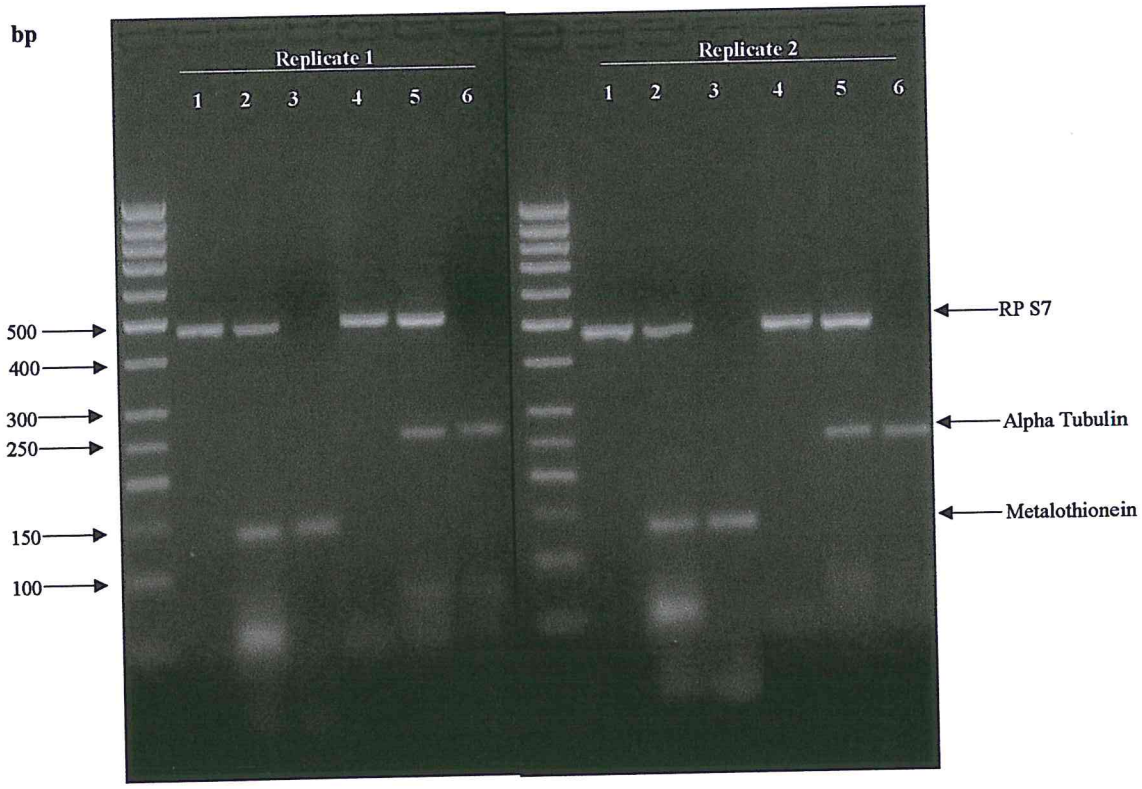

Fig 4: Control for Competition between both Metallothionein and Alpha Tubulin and RP S7 Primer Sets

Lane 1 and 4 represents amplification products of only RP S7 internal Control primer in the absence of competition. Lane 2 represents amplification products of both RP S7 and metallothionein when multiplexed (presence of competition). Lane 3 represents amplification products of only metallothionein, primer in the absence of competition. Lane 5 represents amplification products of both RP S7 and alpha tubulin when multiplexed (presence of competition). Lane 6 represents amplification products of only alpha tubulin, primer in the absence of competition. Additionally, lanes 1-3 were are products of 22 thermocycle PCR while the rest are products of 25 cycles. The products were run on 3% Agarose.

3.3.1.3 Semi-Quantitative RT-PCR

Alpha tubulin expression volumes increased with cadmium selection generations, in a dose dependent manner, differentiating from all other treatments, including control in fifth generation (Fig. 5). The increase was at least 1.5 fold against the other treatments in the fifth generation (Fig 5c). However, expression volumes of alpha tubulin in control and to copper, and lead selection were similar between all generations (Fig. 5). This in fact seemed to be reducing in magnitude with generations, especially between the third (Fig. 5b) and fifth (Fig .5c) generations. The expression levels were significantly ($p<0.05$) different between individual heavy metals, generations and genes. Interactions between heavy metals and generations, as well as between genes and generations were also significant ($p<0.05$), though this was absent ($p<0.05$) between heavy metals and generations.

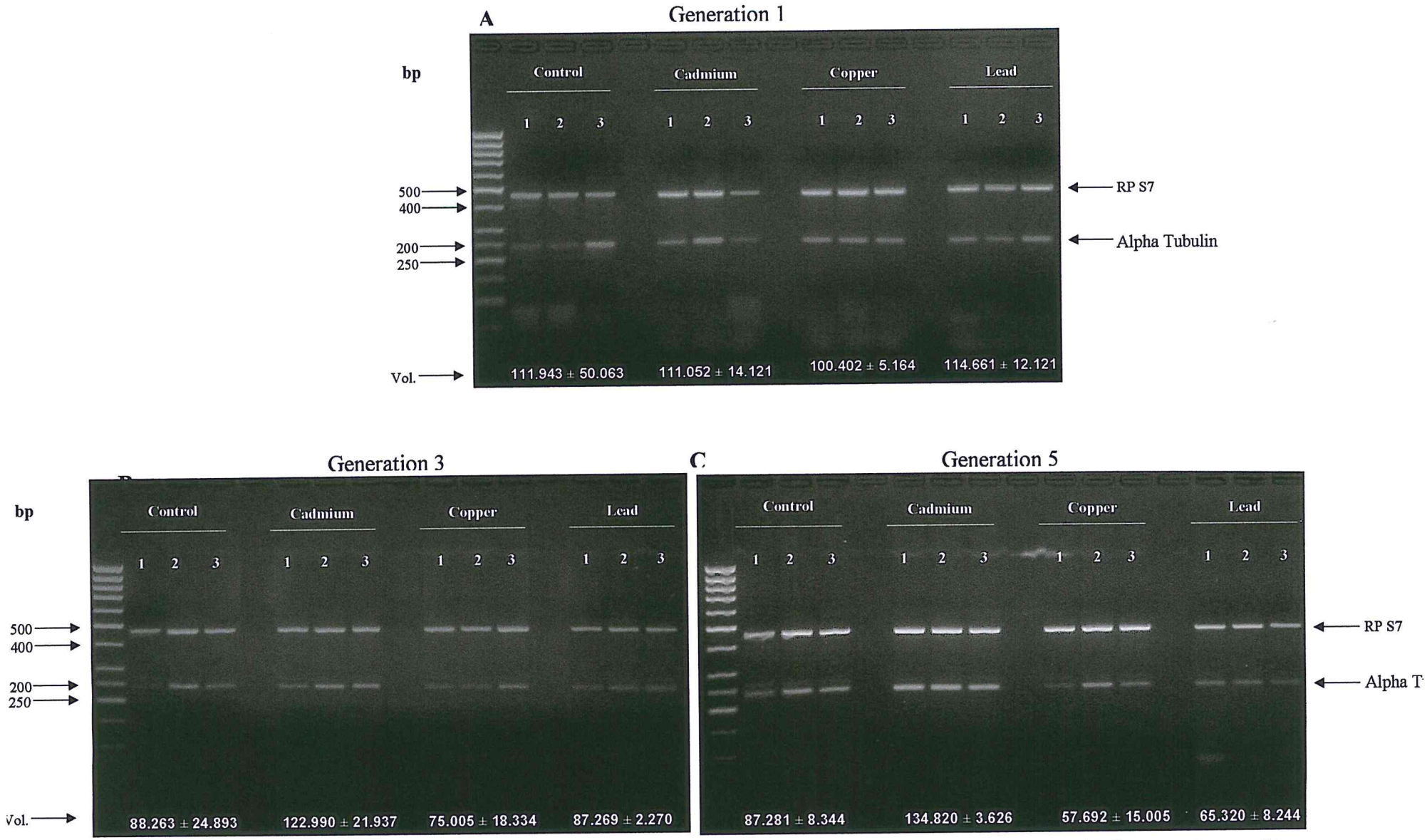

Fig 5: Response levels of Alpha Tubulin Multiplexed with RSP7 Internal Control in Various Generations of Third Instar *An. gambiae s.s* Larvae Control and to Selection by Various Heavy Metals

The lanes 1, 2, 3 represent replicate samples in the same treatment and the results are represents as volume. Volumes (Vol) are alpha tubulin expressions in nanograms (ng) normalized against RP S7 internal control expressions.

Metallothionein expression levels, in response to cadmium selection, were higher than those of all other treatments, including control in all generations (Fig. 6a, b, c). The response increases between the generations, relative to control though not clearly dose dependent. However, metallothionein response volumes to copper selection dose, dependent, which were similar to control in the first (Fig. 6a) before significantly differentiating in the third (Fig. 6b) and more in the fifth (Fig. 6c) generations. Response volumes to lead selection were similar to control in all generations (Fig. 6a, b, c) and marginally appeared to increase with selection relative to the control.

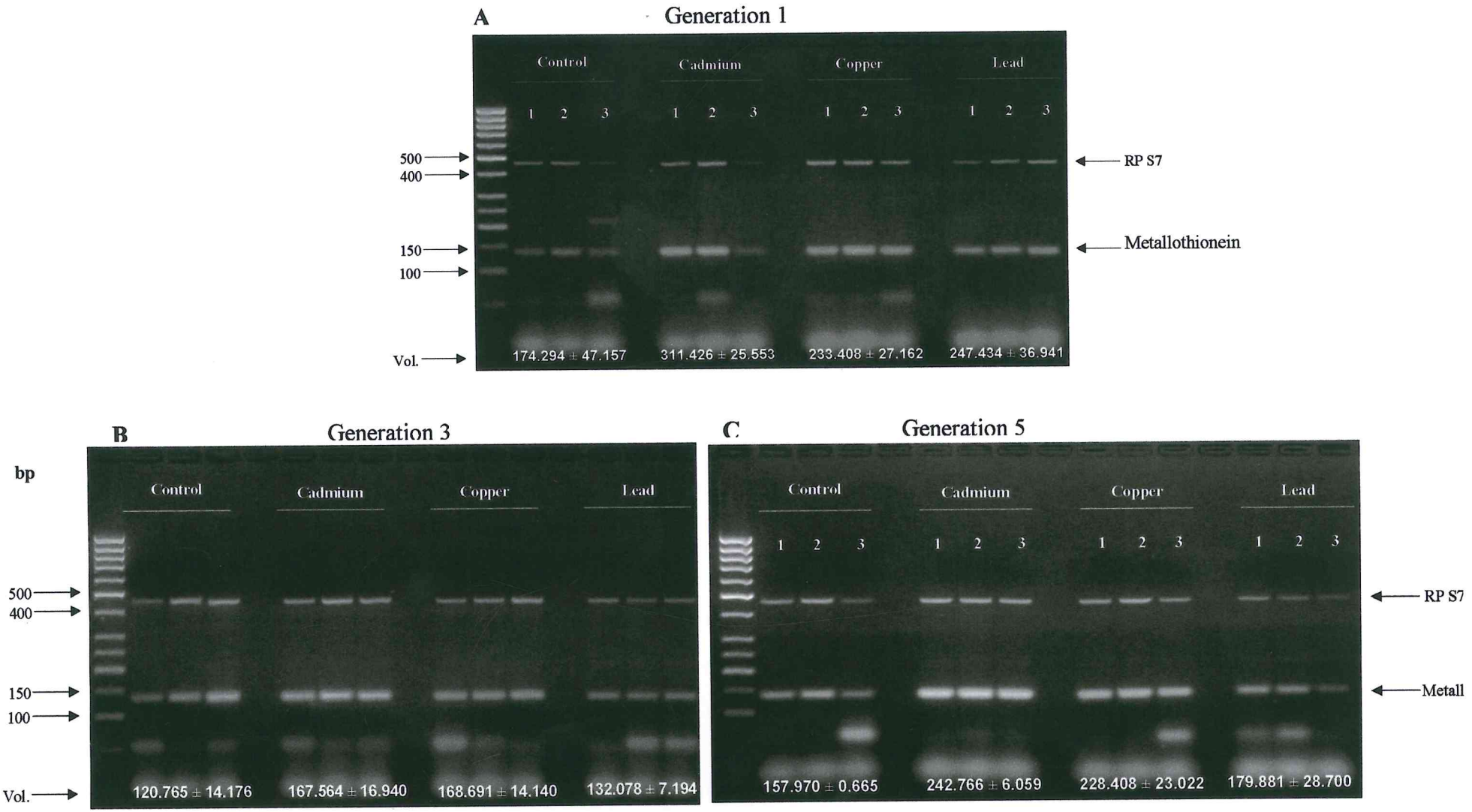

Fig 6: Response levels of Metallothionein Multiplexed with RSP7 Internal Control in Various Generations of Third Instar *An. gambiae s.s* Larvae Control and to Selection by Various Heavy Metals

The lanes 1, 2, 3 represent replicate samples in the same treatment and the results are represents as volume. Volumes (Vol) are metallothionein expressions in nanograms (ng), normalized against RP S7 internal control expressions.

3.4 Two-Dimensional Gel Electrophoresis Analysis of Differential Expression of Proteins in *An. gambiae s.s* Larvae in Response to Selection by Cadmium, Copper and Lead Heavy Metals

Most of the differentially expressed spots had acidic charge and were of low molecular weight among all metals and generations. The proportion of the spots with low pH (acidic) increased with generations except for copper, where the third generation was lower than the first. The proportions were 65.22 (Fig. 7), 64.29 (Fig. 8) and 60.00%(Fig. 9) acidic in first generation, 70.83 (Fig. 10), 68.42 (Fig. 11) and 55.56% (Fig. 12) acidic in the third generation, and 78.95 (Fig. 13), 76.19(Fig. 14) and 78.26% (Fig. 15) acidic in the fifth generation for cadmium, copper and lead respectively. The low molecular weight spots in this category were most expressed in the third generations and least in the fifth respectively. The distributions were 71.74 (Fig. 7), 71.43 (Fig. 8) and 75.00%(Fig. 9) in first generation, 50.00 (Fig. 10), 84.21 (Fig. 11) and 85.19 % (Fig. 12) in the third generation, and 63.16 (Fig. 13), 66.67(Fig. 14) and 56.52% (Fig. 15) in the fifth generation for cadmium, copper and lead respectively. There was no specific trend in distribution of the neutral, basic as well as among medium, and high molecular weight differentially expressed protein spots between metals and generations. Both molecular weight and isoelectric point distribution patterns of both differentially and constitutively expressed protein spots were similar ($P>0.05$). The magnitude of differentially exposed proteins was significantly ($P<0.05$) influenced by interaction between type of heavy metals and generation.

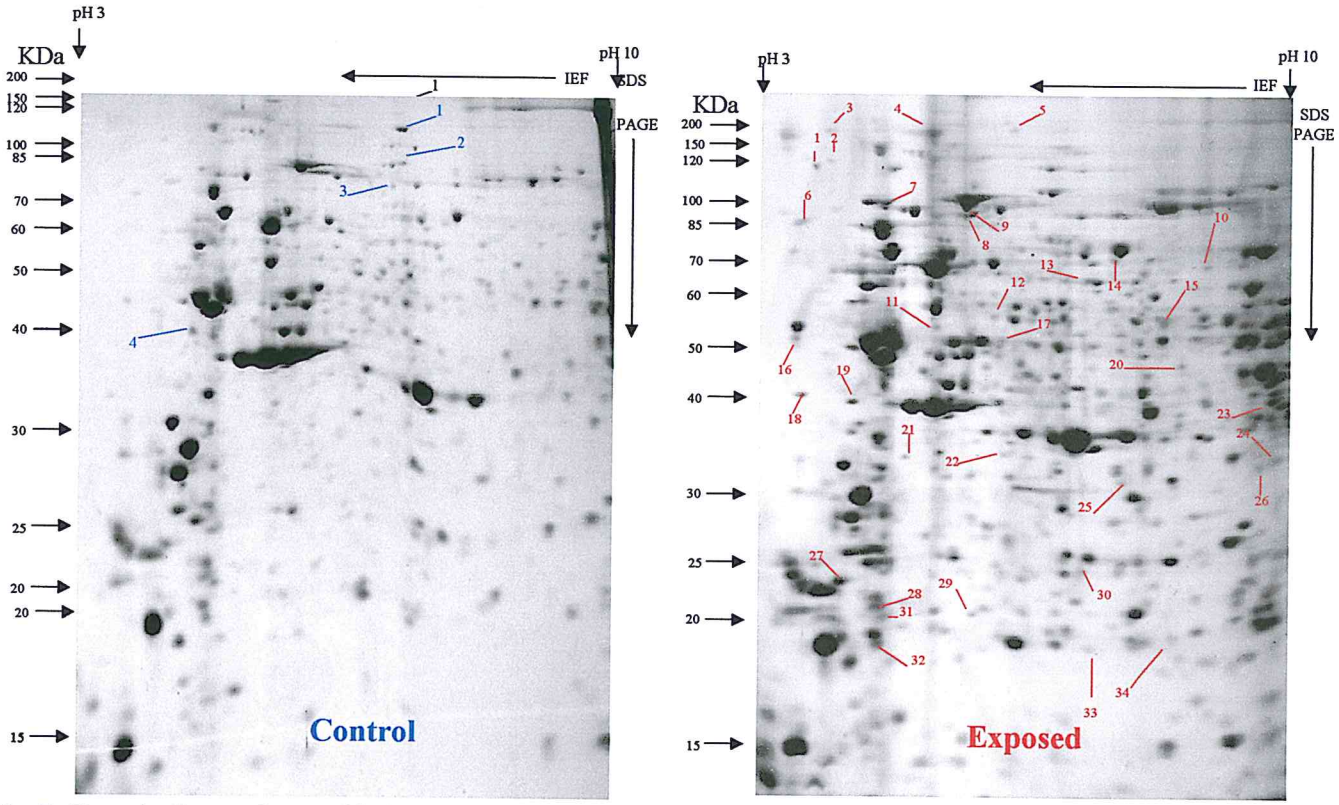

Fig 7: Protein Spots from Third Instar *An. gambiae* Larvae First Generation Non-Selected Control and Cadmium LC_{30} Selection Exposure Survivors

Spots indicated by lines and numbers are differentially expressed in response to cadmium selection in the first generation, consistently in the three replicates. Such spots in the control gel (blue) are down-regulated while those in the exposed gel (red) are up-regulated

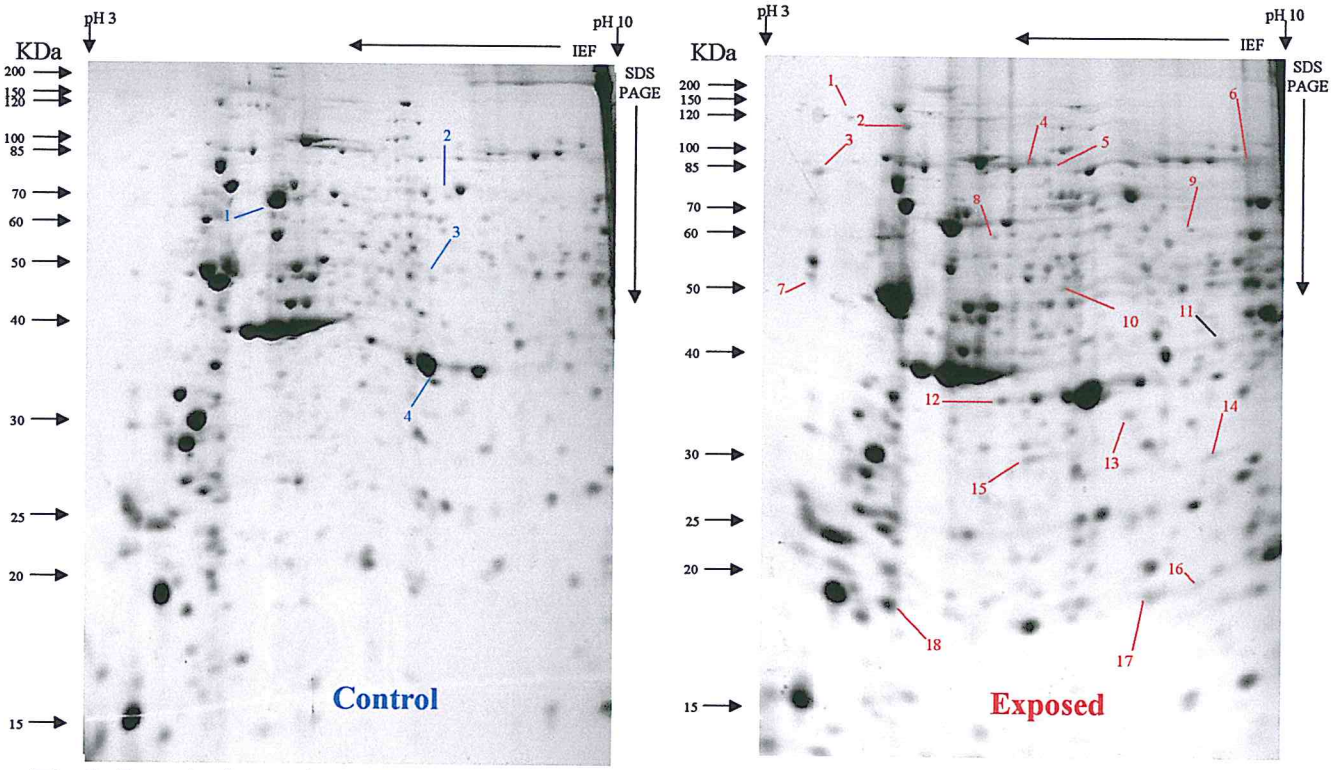

Fig 8: Protein Spots from Third Instar *An. gambiae* Larvae First Generation Non-Selected Control and Copper LC_{30} Selection Exposure Survivors

Spots indicated by lines and numbers are differentially expressed in response to copper selection in the first generation, consistently in the three replicates. Such spots in the control gel (blue) are down-regulated while those in the exposed gel (red) are up-regulated

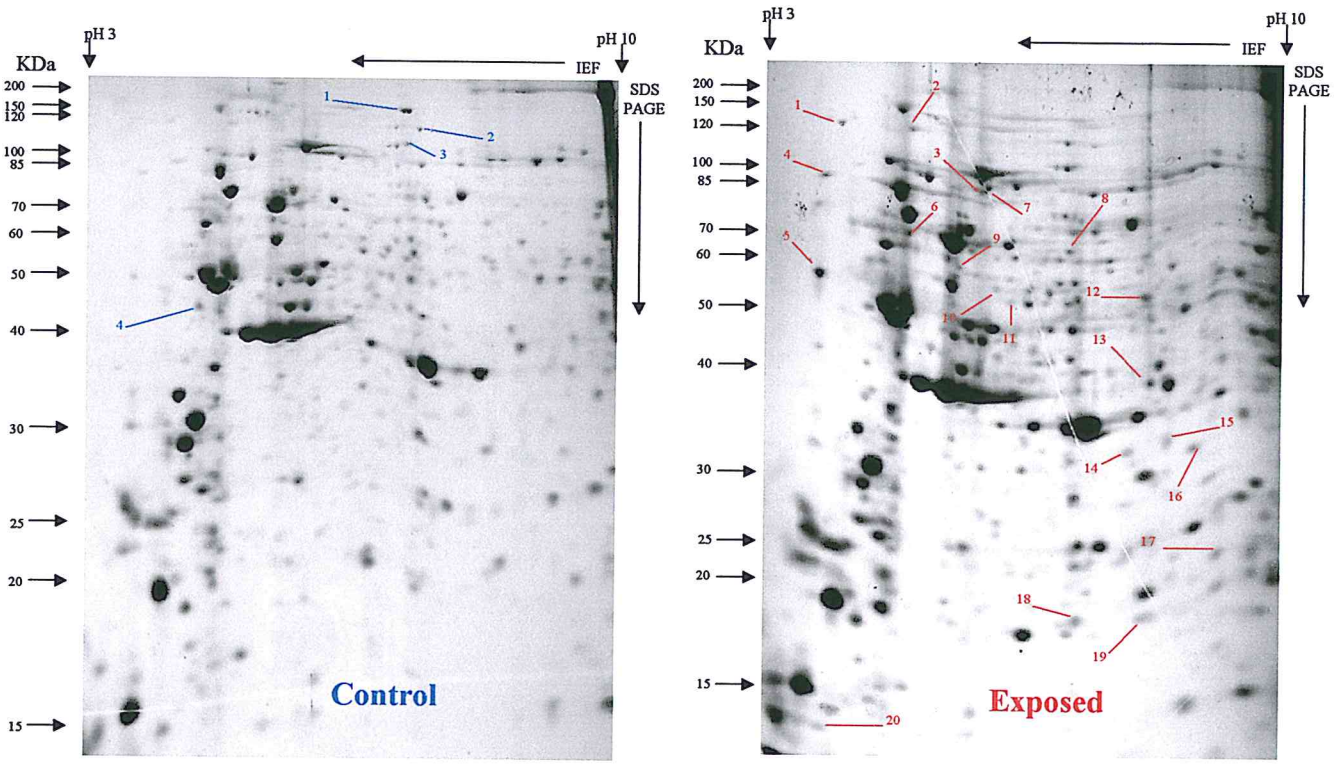

Fig 9: Protein Spots from Third Instar *An. gambiae* Larvae First Generation Non-Selected Control and Lead LC_{30} Selection Exposure Survivors

Spots indicated by lines and numbers are differentially expressed in response to lead selection in the first generation, consistently in the three replicates. Such spots in the control gel (blue) are down-regulated while those in the exposed gel (red) are up-regulated

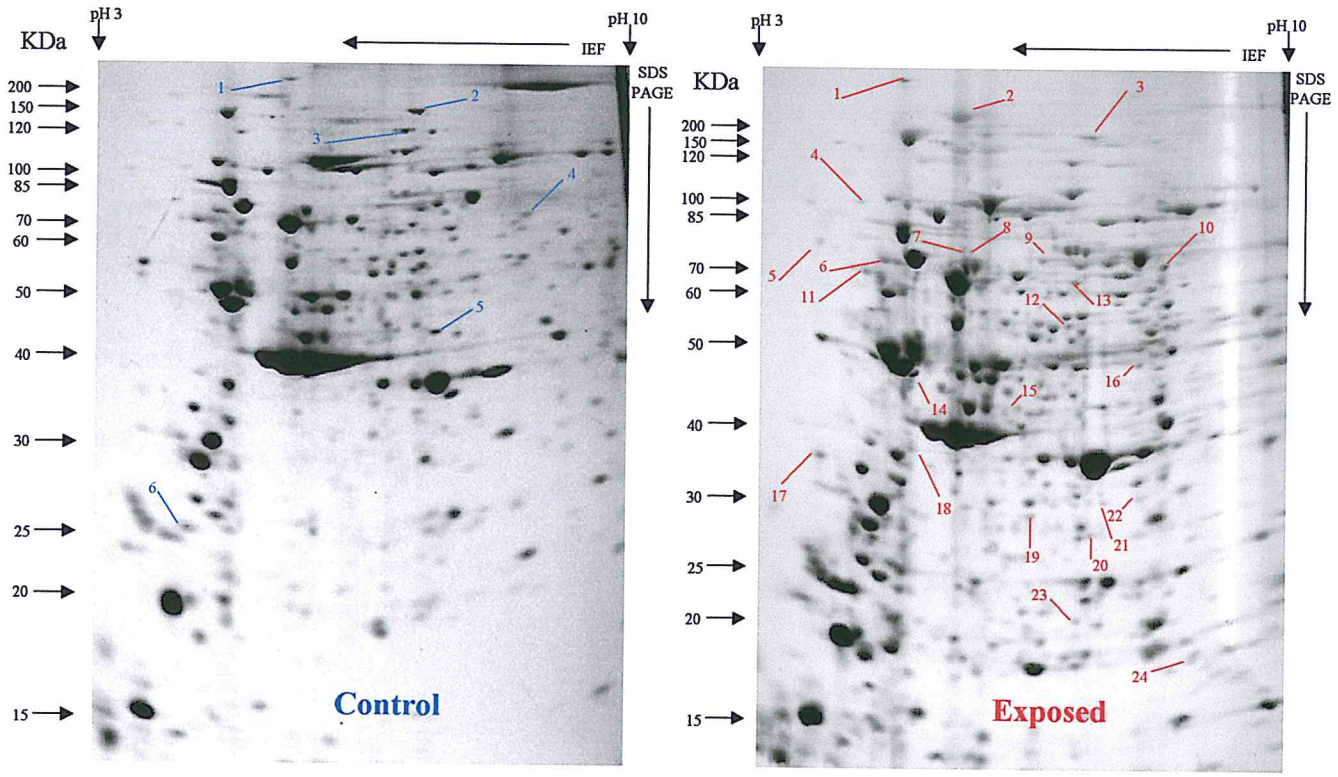

Fig 10: Protein Spots from Third Instar *An. gambiae* Larvae Third Generation Non-Selected Control and Cadmium LC_{30} Selection Exposure Survivors

Spots indicated by lines and numbers are differentially expressed in response to cadmium selection in the third generation, consistently in the three replicates. Such spots in the control gel (blue) are down-regulated while those in the exposed gel (red) are up-regulated

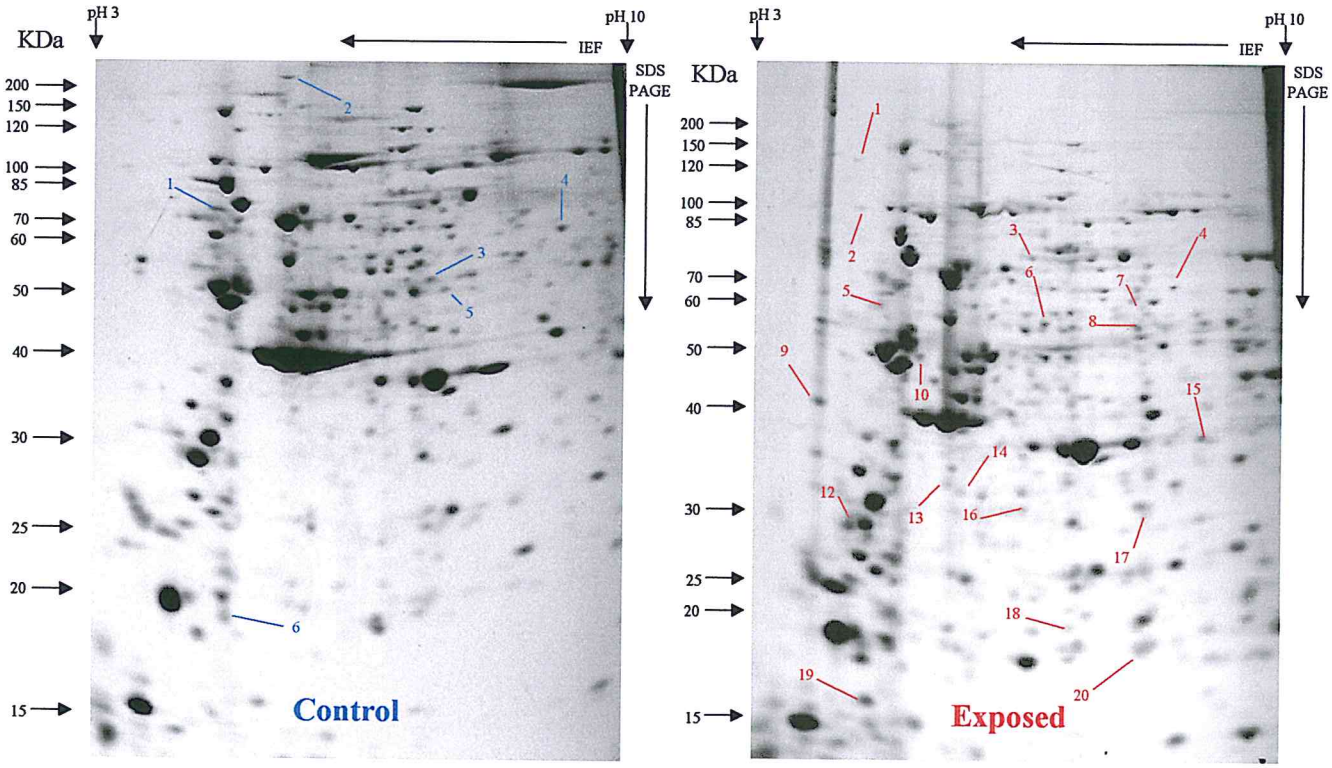

Fig 11: Protein Spots from Third Instar *An. gambiae* Larvae Third Generation Non-Selected Control and Copper LC_{30} Selection Exposure Survivors

Spots indicated by lines and numbers are differentially expressed in response to copper selection in the third generation, consistently in the three replicates. Such spots in the control gel (blue) are down-regulated while those in the exposed gel (red) are up-regulated

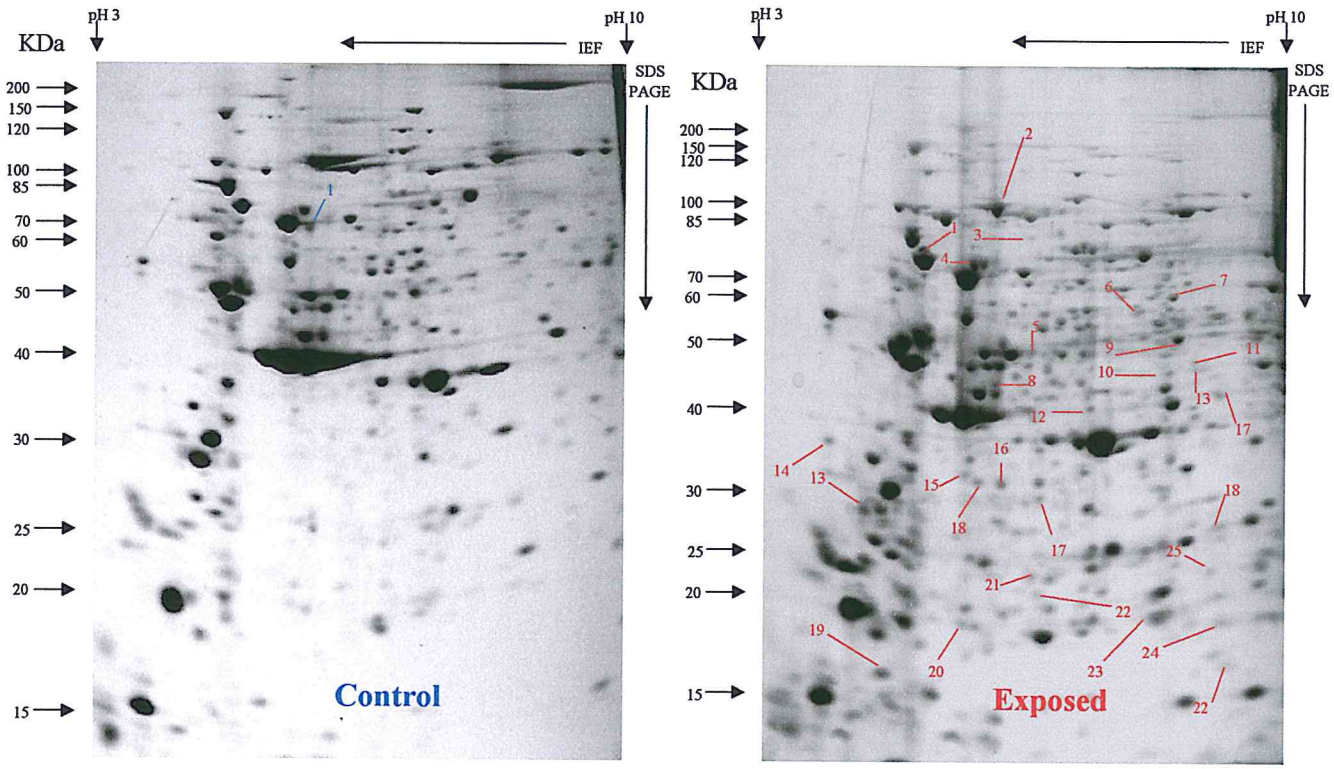

Fig 12: Protein Spots from Third Instar *An. gambiae* Larvae Third Generation Non-Selected Control and Lead LC_{30} Selection Exposure Survivors

Spots indicated by lines and numbers are differentially expressed in response to lead selection in the third generation, consistently in the three replicates. Such spots in the control gel (blue) are down-regulated while those in the exposed gel (red) are up-regulated

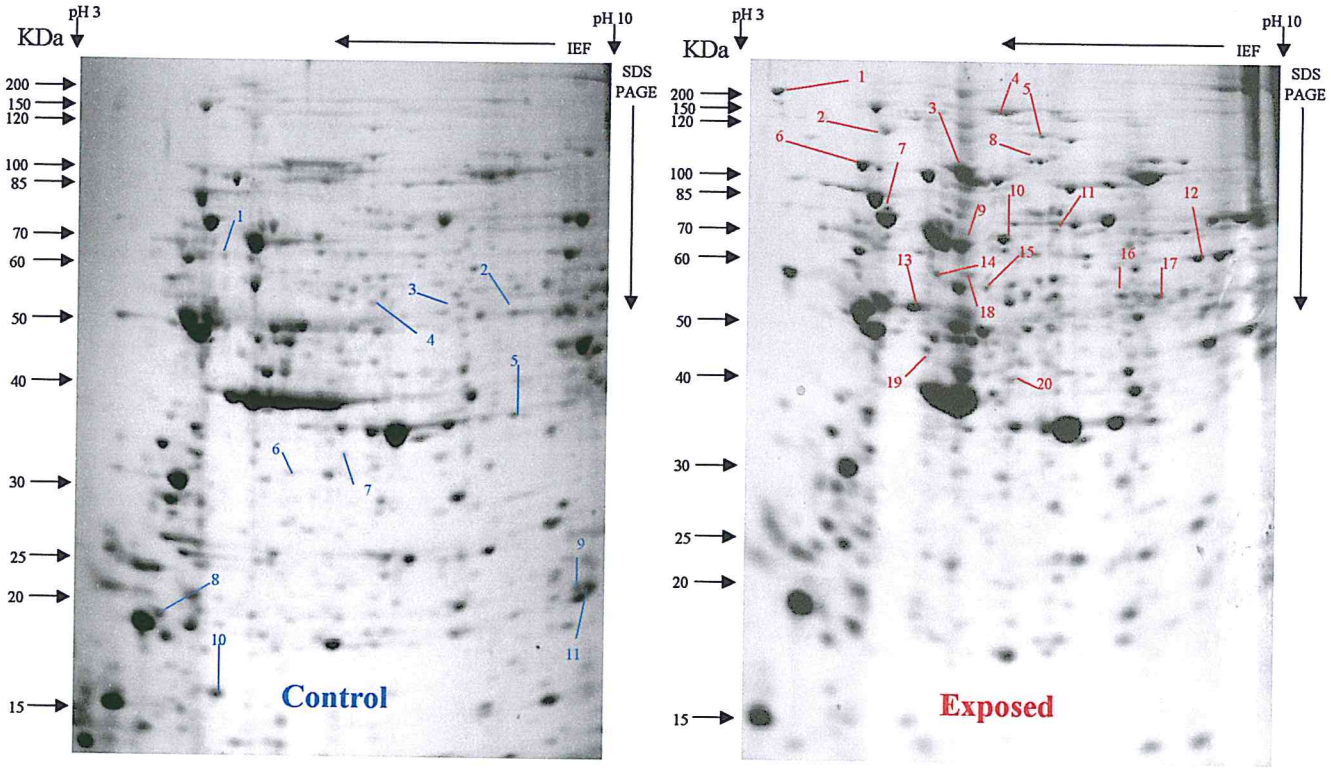

Fig 13: Protein Spots from Third Instar *An. gambiae* Larvae Fifth Generation Non-Selected Control and Cadmium LC_{30} Selection Exposure Survivors

Spots indicated by lines and numbers are differentially expressed in response to cadmium selection in the fifth generation, consistently in the three replicates. Such spots in the control gel (blue) are down-regulated while those in the exposed gel (red) are up-regulated

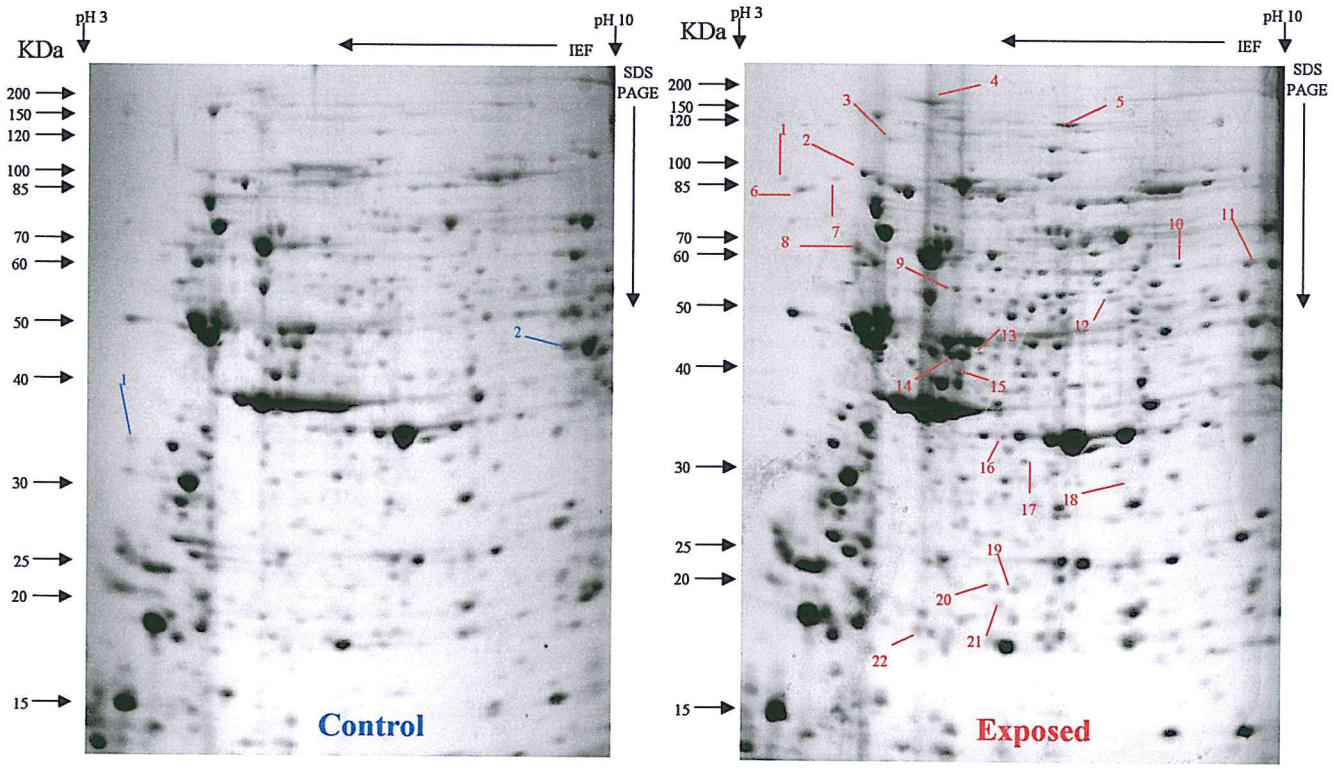

Fig 14: Protein Spots from Third Instar *An. gambiae* Larvae Fifth Generation Non-Selected Control and Copper LC_{30} Selection Exposure Survivors

Spots indicated by lines and numbers are differentially expressed in response to copper selection in the fifth generation, consistently in the three replicates. Such spots in the control gel (blue) are down-regulated while those in the exposed gel (red) are up-regulated

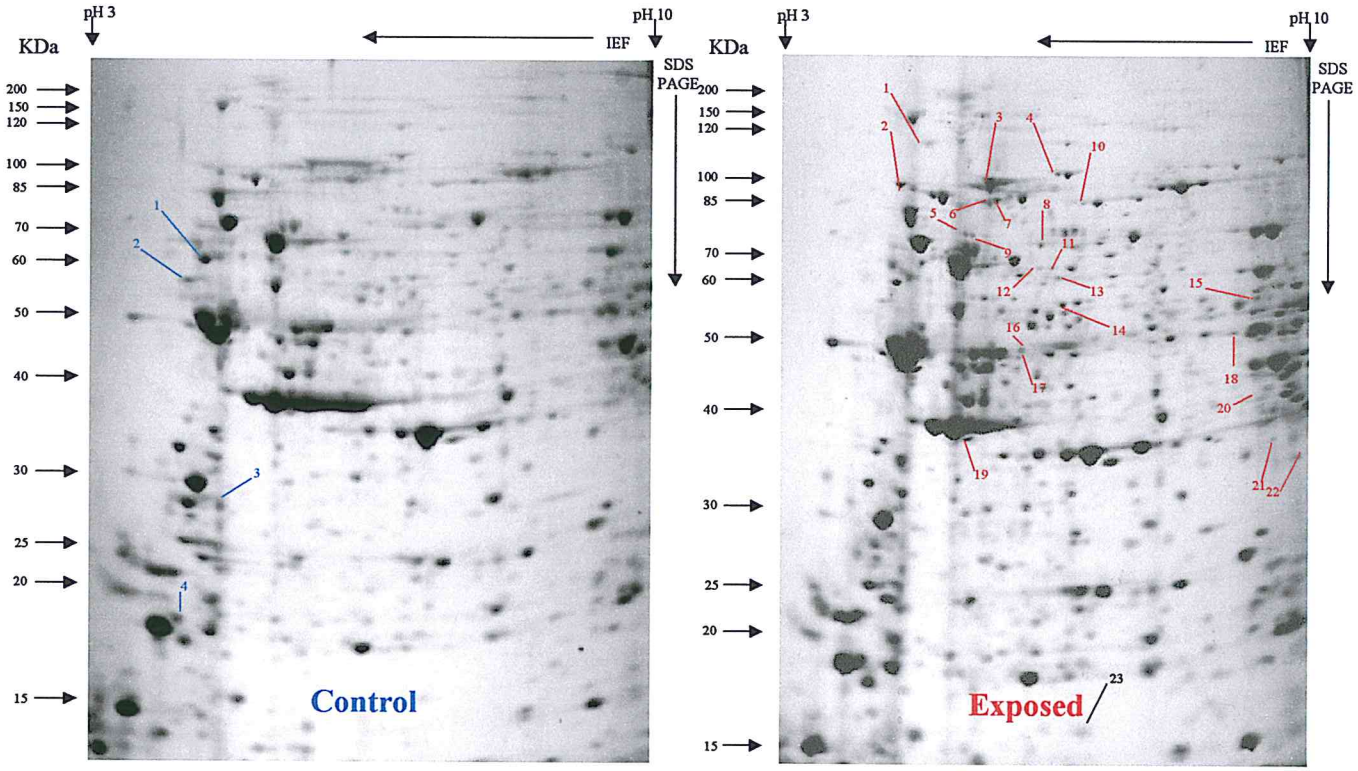

Fig 15: Protein Spots from Third Instar *An. gambiae* Larvae Fifth Generation Non-Selected Control and Lead LC_{30} Selection Exposure Survivors

Spots indicated by lines and numbers are differentially expressed in response to lead selection in the fifth generation, consistently in the three replicates. Such spots in the control gel (blue) are down-regulated while those in the exposed gel (red) are up-regulated

However, the response magnitude to selection among individual metals between generations was only significant ($P<0.05$) in cadmium selection, and was only similar ($p>0.05$) between metals in the first generation (Fig 16). The magnitudes of response of differentially expressed spots in control were similar ($P>0.05$) between generations among metals and there was no significant ($p>0.05$) interaction between type of heavy metal and generation (Fig 17)

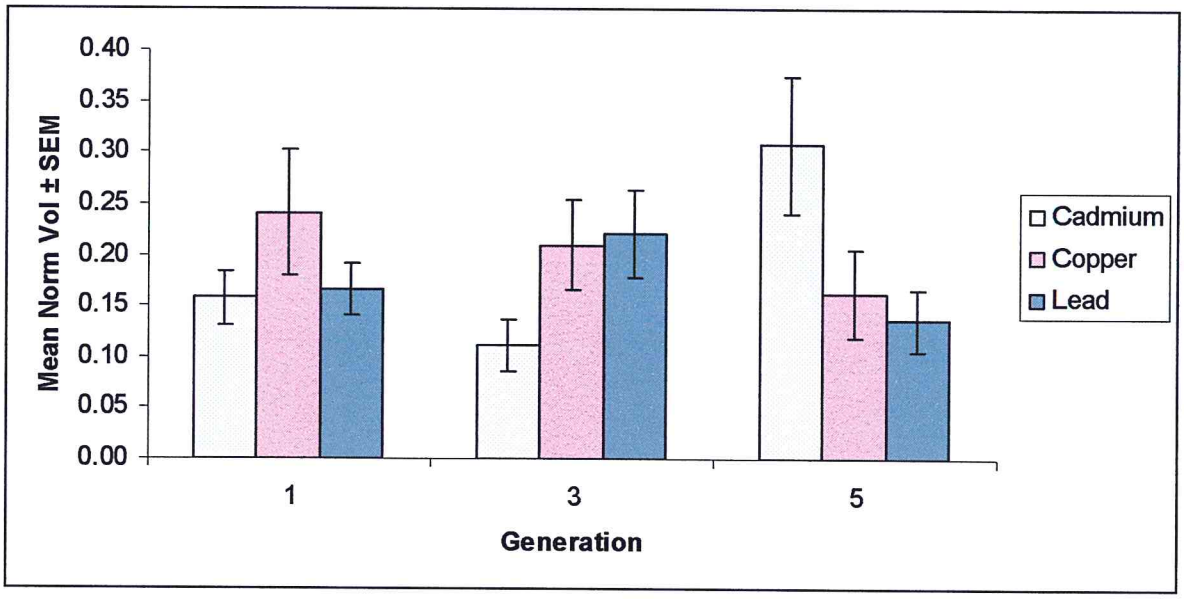

Fig 16: Protein Profiles in Third Instars *An. gambiae* Larvae Differentially Expressed in Response Various Heavy Metals Selection at Various Generations

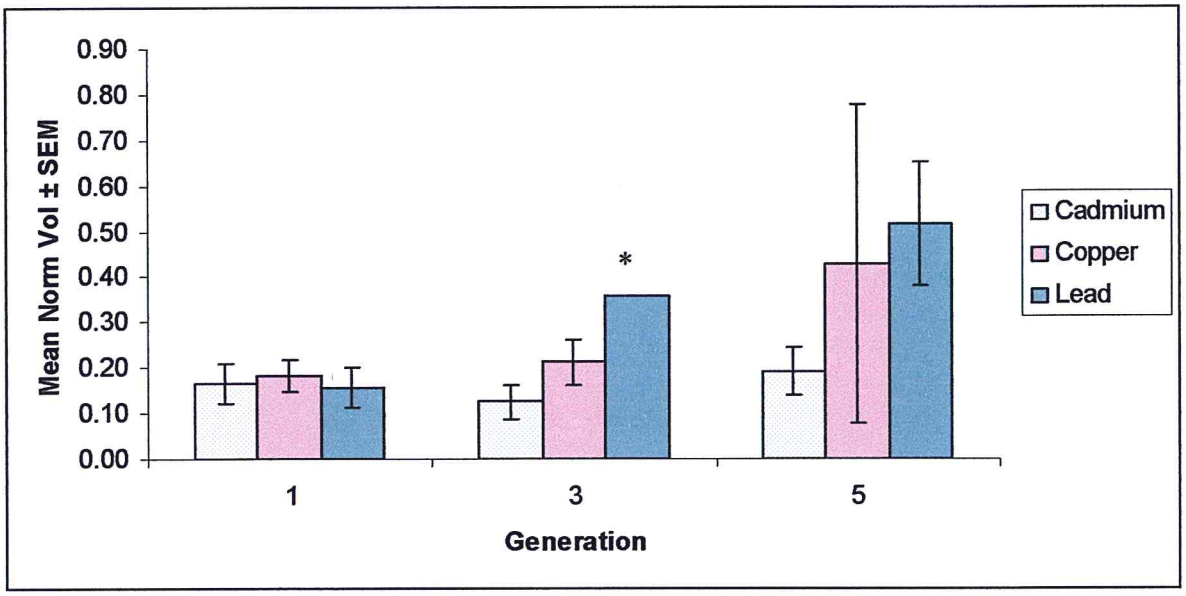

Fig 17: Protein Profiles in Differentially Expressed Third Instars *An. gambiae* Larvae of Various Heavy Metals Selection at Various Generations

* SEM not performed. Fewer than two cases observed

Table 8 illustrates distribution patterns of constitutively expressed protein spots following various treatments in different generations. Briefly, there was significant ($p<0.05$) variation in pattern of magnitude of constitutively expressed spots among generations in both cadmium and copper while this was not the case ($p<0.05$) in lead selection. The spots were most up-regulated in the third and fifth generations in copper and cadmium selections respectively, and down regulated in the first generation of both metals. The pattern was similar ($P>0.05$) in the first generation, but not in the third and fifth generation ($p<0.05$) among the metals within generations. Copper and cadmium had most up-regulated spots in the third and fifth generation respectively, while lead and copper had most down regulated spots in the two generations respectively.

Table 8: Percentage Distribution of Patterns of Common Protein Spots between Non-Exposed Control and Heavy Metal Exposed Third Instar *An. gambiae* Selection in Various Generations

	Generation 1			Generation 3			Generation 5		
	Cadmium	Copper	Lead	Cadmium	Copper	Lead	Cadmium	Copper	Lead
n	162	131	193	210	154	187	44	163	216
Up-regulated	27.16	22.14	29.53	24.76	43.51	34.22	61.36	31.29	32.41
Unchanged	44.44	48.85	48.19	48.10	37.66	34.22	20.45	54.60	48.15
Down-regulated	28.40	29.01	22.28	27.14	18.83	31.55	18.18	14.11	19.44

Chapter 4

Discussions, Conclusions and Recommendations

4.1 Discussion

4.1.1 Heavy Metals and their Impact on Mosquito Larval Habitats in Urban Kisumu and Malindi, Kenya

This study has provided insights into the water and soil chemistry, as well as mosquito larval ecology in potential larval habitats during the dry seasons in Kisumu and Malindi. Prevalence of some of the heavy metals in the surface waters of habitats in both cities were above the WHO acceptable limits for drinking water with, exception of chromium, copper, iron, lead, manganese and zinc in Malindi. WHO recommended levels in dinking water for cadmium, chromium, copper, iron, lead, manganese and Zinc are 0.003, 0.050, 2.000, 2.000, 0.010, 0.500 and 3.000 ppm respectively (WHO, 1996). The heavy metals were higher than waters of Lagos lagoon (Okoye, 1991). The reverse was observed with regard to the soil sediments (Okoye *et al.,* 1991). The observed differences are attributable to dilution, continuous water exchanges and sedimentation in the lagoon (Okoye, 1991) and concentration of the heavy metals in the stagnant habitats in the Kenyan cities by the tropical sun during the dry season when the study was done. High levels of pollution are evident in larval habitats in both cities, and could significantly be increasing

Differences in pollution between habitats in Kisumu and Malindi are attributable to natural and anthropogenic heavy metals. The natural contribution may be through inherent composition of soil substrate constituting basal geographic land profile in the cities. The cities are more than 1000 km apart and are further separated by the Great Rift Valley. Contribution of Sea Spray (Hutton, 1983) to the heavy metal deposits in the

habitats cannot be discounted since these are riparian cities. Anthropogenic sources of the heavy metals are probably the major contributors. These include atmospheric pollution from waste incineration and fossil fuel combustion, releasing about 20 toxicologically important metals in the environment, including, chromium, copper, manganese, zinc, cadmium, lead and nickel as global aerosols (Chang and Cockerham, 1994; Nriagu and Pacyna, 1988; Korte, 1983). Others sources include industrial waste from paints, pigments, batteries and plastics manufacture or discarded products, and domestic sewage waste. Among anthropogenic sources, domestic sewage waste and oil wastes from car washes and repairs are probably the most significant contributors of the heavy metals pollution observed. It may be noted that industrial activity is low in both study areas.

Most of the heavy metal detected in the surface water were anthropogenic, transient and were coupled with temporal heterogeneity of the concentrations of each of the heavy metals. This is underscored by absence of correlation between concentrations of each of the heavy metals in the water and sediments. High correlations could have indicated natural occurrence, permanence and homogeneity in heavy metal concentrations in the habitats since under natural conditions, they originate from the sediments and are released to the surface water or was introduced into permanent non-flowing habitats, following which excess heavy metals would have settled on the bottom sediments. Either way, there would have been a good correlation between concentrations of the heavy metals in the sediments and in the water. In stable environments, sediments serve as a repository of heavy metals in aquatic systems, sometimes accounting for 99% on the total amount (Renfro, 1973). The heavy metals had a common source as evidenced by high

correlation between levels of most of the metals, with higher correlations among the concentrations in water than in sediments again emphasizing the anthropogenic and transient nature, and temporal differences. Most of the heavy metals in the larval habitats were therefore anthropogenic, with common sources including domestic sewage discharge and products of petrochemical combustion whose compositions are generally homogenous.

Planning and drainage did not significantly influence spatial distribution of heavy metals suggesting that the factors responsible for heavy metals pollution in both cities transcended planning and drainage limitations and are ubiquitous. The most probable candidates once again include domestic sewage effluent and airborne products of petrochemical combustion. Elevated concentrations of copper, iron and zinc at unplanned, well drained sources indicate a localized pollution source and can be attributed to localized activities of commercial or industrial nature. Slight variations in heavy metal concentrations between natural and anthropogenic habitats, with consistently higher concentrations in the latter suggest possible inter-linkage between the habitats, with higher levels in the anthropogenic habitats. This phenomenon can also be attributed to common source of most of the pollutants, such as ubiquitous airborne products of petrochemical combustion and domestic sewage discharge

Lower correlation between concentrations of some of the heavy metals and presence of *An. funestus, An. gambiae*, culicines of *Aedes aegypti* mosquitoes indicate that the concentrations of the heavy metal in the habitats do not adversely affect the mosquito

larvae, suggesting that the mosquito larvae are tolerant or adapted to proliferating in such concentrations, especially anophelines following chronic exposure. This notion is underscored by observations that mean heavy metal concentrations of metals such as cadmium, in anopheline containing habitats are within the 0.098 ppm level, the threshold chronic concentration levels established for *An. gambiae* under laboratory conditions. Alternatively, possible antagonistic effects of the heavy metals among themselves and with other contaminants might have also reduced bioavailability of the heavy metals to the mosquito larvae. The larvae are capable of withstanding high iron, zinc and copper concentrations since these metals are essential bio-molecules, becoming toxic only at much higher concentrations (Hare, 1992), which may also be responsible for the positive association between copper concentration and *Ae. aegypti* presence in water from larval habitats in Kisumu. Culicines are more adaptive to pollution, especially organic pollutants (Subra, 1981) than many other mosquito species. Therefore, the mosquitoes seem to have developed a mechanism for countering the heavy metal toxicity in their habitats, especially in the dry season when there is concentration of the heavy metals in the habitats due to evaporation.

4.1.2 Biological Cost of Heavy Metals Resistance in *Anopheles gambiae ss*

These findings demonstrate that *An. gambiae* ss, the principle vector on malaria in Sub-Sahara Africa, can rapidly adapt to heavy metals exposure/challenge, as exhibited by dramatic increase by more than 10 fold in heavy metals resistance over a span of only five generations. This observation is particularly interesting, since this is contrary to the classical dogma that anophelines are sensitive to pollution and proliferate in relatively

unpolluted aquatic habitats (Gwadz and Collins, 1996). These findings negate this dogma and probably provide insights into the adaptation process that mediated proliferation of anopheline observed in polluted habitats in Kinshasa, Zaire (Coene, 1993) and man-made aquatic habitats in Accra and Tema, Ghana (Chinery, 1984; Chinery, 1995).

The development of tolerance is facilitated by lower (LC_{30}) selection pressures than is traditionally applied (LC_{50}) in selection studies, since with the latter selection regime, none of the larvae survivors emerged as adults, probably due to delayed toxicity. In light of this observation, it can be hypothesized that adaptation of *An. gambiae ss* to heavy metal polluted habitats would most likely favor populations of *An. gambiae ss* that are indigenous to the urban areas with higher ($>LC_{30}$) heavy metals pollutions, than those migrating from relatively unpolluted peri-urban and rural areas. This is because it can be assumed that the former co-exist with the gradual changes in heavy metals pollution of the habitats, and hence gradually adapt. Similarly, a rapidly urbanizing region, concomitant with high heavy metals population, can significantly reduce *An. gambiae* population density through selection, and may partially be responsible for the significant reduction in mosquito species diversity in Accra and Tema, Ghana (Chinery, 1995), Burkina Faso, Benin (Coluzzi, 1993), Kinshasha, DRC (Coene, 1993) and Brazzaville, Congo (Trape and Zoulani, 1987a). However, empirically, the magnitude of interaction among heavy metals as well with other pollutants and components in the habitats may significantly affect the process of adaptation, which may thus differ from those observed here.

Mechanistically, the resistance to heavy metals may be happening at both physiological and genetic levels. Physiologically, the resistance may be due to reduced bio-availability of the heavy metals through intracellular compartmentalization and sequestration, heavy metal toxicity inactivation by binding to metallothionein (Hare, 1992), heat shock proteins (Abe *et al.,* 1994) and glutathione (Singhal *et al.,* 1987) or apoptosis to eliminate damaged cells (Habeebu *et al.,* 1998). Genetically, the observed resistance could be due to selection for alleles conferring resistance to heavy metals among the sample populations, or to a modification of the pre-existing gene to those that confer the resistance, since such alles are rarely synthesized *de novo* (Dujon *et al.,* 2004). Physiological mechanism may thus be operating generational resistances levels while genetic mechanism may be operational at both intra and inter generational levels since physiological mechanisms are reversible and non-inheritable, while genetic mechanisms are heritable (Klerks and Weis, 1987; Okey, 1992).

The adaptation/resistance to heavy metal exposure comes at an enormous cost to *An gambiae ss* in heavy metal-free environments, and is empirically possible due to the heterogeneous composition of aquatic *An. gambiae* habitats. The resistance trait compromised egg viability in a heavy metal independent manner, suggesting that resistance would empirically reduce emergent *An. gambiae ss* populations in nature and that the molecular mechanisms mediating such reduction in viability of the eggs in the different heavy metal resistant populations are under similar regulatory process. The process could be adversely affecting egg viability by retarding egg maturity or fertility. Thus, identification of the genetic factors/mechanism responsible for fertility in *An.*

gambiae ss might provide more insights on the underlying process mediating the reduction in egg viability following heavy metals selection. Despite the reduction in egg viability, the egg stage had overall least contribution toward the juvenile generational mortality as shown by the killing factor, though the impact was significantly lower in the control than in the heavy metals selected populations.

Larval mortality was singly the largest juvenile generational killing factor, responsible for the drastic reduction in subsequent emergent adults population among the non-selected control and heavy metals selected populations, and the impact was several fold higher in the latter than in the former. The high killing factor at this stage can partially be attributed to natural mortality coupled to cannibalism of relatively younger larvae by older ones (Koenraadt and Takken 2003). Despite that possibility, the heavy metals resistance also seems to further significantly reduce the magnitude and rate of larval survivorship, generally in a heavy metal dependent manner, relative to the control. The lower effect of resistance to copper on larval mortality, than the other heavy metals may be attributed to lower copper toxicity than lead and cadmium to insects. This is because copper mediates functionally important biological process in insects at a lower physiological concentration (Hare, 1992), while cadmium and lead are more toxic and have no known essential biological function (Beyersmann and Hechtenberg, 1997; Bouton and Pevsner, 2000). Empirically, the increased number and rate of larval mortality would reduce pupation and emergent adult, and complement effects of other predators of juvenile *An gambiae*, such as including dragon fly nymphs of the *Chorixidae* family and tadpoles (Beesley, 1973) larvivorus fish such as *Gambusia, Poecilia* and *Tilapia* spp, *Ctenopharyngodon idella*,

and parasitic nematode *Romanomermis culicivorax* and *R. xyangari* (Singh *et al.*, 1977) in reducing *An. gambiae* population.

The lower magnitude of pupation in the heavy metals selected than in non selected population may partially be due to similarly lower preceding larval survivorships and the higher killing factor impacting the heavy metals selected populations. However, the delayed pupation in cadmium and copper selected relative to the control populations suggests that the resistance would prolongs the aquatic stage of the life cycle of *An. gambiae,* hence predispose them to more predation and reduce their natural population increase.

The similarity of the sex ratio in emerging male and female population among the non-selected and heavy metal selected populations, suggests that the potential effective population size, which determines the populations characteristic intrinsic rate of increase was not affected by the treatments. However, the heavy metal appears to reduce male and female emergence in a heavy metal and non-heavy metal dependent manner, respectively, though the rate of emergence was not significantly affected by selection in both sexes. The selection did not also appear to affect the size of emerging females adults as indicated by similar wing lengths among treatments.

Both male and female adults in control and heavy metals selected populations displayed characteristic Type I mortality curves, characterized by increase in mortality at old age and hence the selections do not significantly affect *An. gambiae* vectorial capacity. In

the same light, similarity in life expectancy at 10 days of age between heavy control and heavy metals selected populations suggest that the vector competence would not be significantly affected by the selection, relative to control. However, among heavy metals selected populations the copper selected mosquitoes however seemed to have a higher life expectancy at 10 days than cadmium selected populations, suggesting that among heavy metals selected populations, copper selected populations would be more vector competent, and hence influence local malaria epidemiology. This observation may once more be attributable to the essential nutritional nature of copper to insects in low concentrations (Hare, 1992).

The high (>>1) net reproductive rate among control and heavy metals selected populations suggested that the populations show progressive increase (Elkinton, 1993), though the adapted populations have a reproductive rate in the order of about 10 times less. This suggest that in nature, the non-selected populations are better adapted and would out-compete the heavy metal resistant populations and hence reduce their presence, which in turn will only be established at low populations. However, empirically, this cost and other costs of resistance may ultimately be compensated for by compensatory mutations (Levin *et al.,* 2000), for the population to stabilize in the natural variable environments. The reduction the net reproductive rate may be attributable to physiological stress load imposed by the resistance trait in the heavy metal selected population, which might have adversely affected the *An gambiae ss* reproductive competence. The competence could have been compromised through heavy metal poisoning of the gonadal function, or may be a consequence of re-direction of the

metabolic resources, especially proteins and energy, towards the synthesis of secondary metabolites conferring tolerance to the heavy metals or bioprocessing of these metabolites.

The insignificant effect of the heavy metals selection on mean generation time in selected populations relative to non-selected controls suggests that both adapted and non adapted populations attained maturity at about the same time and followed similar reproductive patterns. Given that the net reproductive rate is a factor of the development time, therefore the heavy metal selected individuals have delayed mortality effects, as indicated by the mortality trend, *k*- values, or most of their eggs would be laid towards the end of adulthood, in a narrow window of time, which gives a higher product of the stage specific fecundity estimate. Delayed oviposition, reaching a peak within a narrow window of time, exposes single cohorts to greater environmental risk to survival or of extinction/ population collapse, due to greater competition within the cohort.

The significant reduction in intrinsic rate of natural increase in the heavy metals selected, relative to the none selected control populations suggests that the resistance to heavy metals once again confers a general reproductive load on the selected populations since the intrinsic rate of natural increase is a factor of the reproductive effort and age specific reproductive effort. Furthermore, due to the progressively increasing nature of the population, the empirical magnitude of the load will depend on the availability of competing populations and aquatic environmental niches polluted with the respective heavy metals for exploitation by the resistant populations.

The ratios of birth and death rate (b/d) and rm/b used to depict relative theoretical colonizing ability is much higher than estimated for *A stephensi* and *A culicufascies* (Reisen and Mahmood 1980). Importantly the difference in both ratios between the heavy metal selected and control populations is not significant. Thus while heavy metal resistance may exert a physiological and reproductive load on the populations, their colonizing ability and competitive advantage may not be significantly affected. In ecological terms, it is possible to hypothesise that heavy metal resistance creates populations that will equally competently exploit the heavy metal polluted environments leading to their gradual adaptation to heavy metal polluted habitats in the urban environments, under high heavy metal selection pressure. This would also occur in the emerging in areas where anophelines were not hitherto important vectors. The heavy metal resistant *An gambiae ss* strain can very easily expand its niche into other extreme environments unrelated to heavy metals presence, and be a potent evolutionary agent (Laland *et al.,* 1999) since adaptation to one stress leads to long term higher fitness levels in the presence of an unrelated stress (Reed *et al.,* 2003). The overall observed cost of heavy metals resistance in *An gambiae ss* may be associated with maintaining the genes for heavy metal resistance or an increased frequency of deleterious mutations in case of modifications of pre-existing genes (Mukai *et al.,* 1972; Fry 2001; Zeyl *et al.,* 2001).

4.1.3 Analysis of Responses of Metallothionein and Alpha Tubulin Genes in *Anopheles gambiae sensu stricto* by Semi Quantitative RT PCR to Selection by Heavy Metals

Contemporary bioinformatic tools were applied to identify heavy metal responsive alpha tubulin and metallothionein gene functional homologues from *Chironomus tentans* (Mattingly *et al.*, 2001) and *Drosophila melanogastaer* (Maroni *et al.*, 1987) respectively in *An. gambiae s.s* and design *An. gambiae* specific primers. The primers were successfully applied in semi-quantitative RT PCR (Marone *et al.*, 2001) to establish expression responses of these genes to heavy metals selection in several generations. However, no changes in responses of both alpha tubulin and metallothionein to lead between generations was detect, attributable to either low sensitivity of the semi quantitative RT PCR or statistically insignificant absolute expression of the affected genes and generations. Despite this limitation, the results suggest that the method can be a relatively rapid and economical approach of molecular screening of large *An. gambiae* larval population for adaptation and bio-monitoring of some heavy metals environmental prevalence in urban environments, where *An gambiae s.s* larvae are environmentally ubiquitous, supplementing other traditional arthropod indicators such as May-fly. Other relatively accurate competitive PCR demands design and synthesis of specific internal competitor for each specific individuals (Ali *et al.*, 1997; Corey and Corey, 1998.), relatively large amount of cDNA and amplification reactions per sample (Marone *et al.*, 2001). Real-time PCR requires a dedicated instrument generally unavailable in standard laboratories (Marone *et al.*, 2001). *An. gambiae s.s* larvae rapidly adapt physiologically to heavy metals challenge as indicated by the several fold increases in tolerance during

selection over five-generations. However, the underlying physiological and/or genetic mechanism behind the rapid increase between third and fifth generations in cadmium selection and third and fifth generation in the rest of the metals is not clear. The resistance/ tolerance was both metal and concentration dependent, as previously observed in *Aedes aegypti* larvae responses to cadmium and copper (Rayms-Keller *et al.,* 1998).

Alpha tubulin appears to be only cadmium sensitive and specific to third instar *An. gambiae s.s.* among the metals evaluated. The same gene was similarly induced in *C. tentans* in response to cadmium challenge, indirectly possibly through calmodulin-mediated signaling pathway (Mattingly *et al.,* 2001). Cadmium and lead activate cadmodulin-mediated pathway by substitution of calcium in binding to calcium binding sites (Behra and Gall, 1991; Goldstein and Ar, 1983). This induction was however not evident by lead and copper probably due to differences in their toxicity mechanism and /or potency in third instar *An. gambiae s.s* larvae. Alpha tubulin is primarily a housekeeping gene (Eisenberg and Levanon, 2003) and is therefore less amenable to effect by many xenobiotics. Additionally, effects of lead on gene expression via calmodulin are not as dramatic as via other pathways such as Protein Kinase C (PKC) probably because 100 fold more lead is required for calmodulin induction relative to PKC (Goldstein, 1993). Most effects would therefore be elicited via the PKC pathway. This in turn suggests that alpha tubulin may be applicable in detection of higher lead prevalence in habitats or where there is higher selection pressure.

On the other hand, responses of metallothionein are consistent with expectation since earlier induction of metallothionein is expected, as constituent vanguard molecules, including glutathione (Singhal *et al.,* 1987), against broad-spectrum heavy metals challenge. The sharp drop in metallothionein expression in the third generation could be attributed to developments of additional physiological or genetic heavy metal adaptations, reducing heavy metal pressure on metallothionein. With respect to cadmium, the initial relatively lower cadmium selection pressure in the first generation could have provided protection against cadmium-induced toxicity, hence the lower response in the third generation. Low-concentration cadmium pretreatment has been shown to provide protection against cadmium-induced genotoxicity in TRL-1215 cells (Coogan *et al.,* 1994). Additionally, copper is an essential element, becoming toxic only at elevated levels (Hare, 1992), hence most copper challenge in the first generation may have been normally metabolized, with any additional eliciting glutathione response or metallothionein response within undetectable limits. Glutathione provides first line of defense against cadmium toxicity before induction of metallothionein synthesis (Singhal *et al.,* 1987; Chin and Templeton, 1993). The lack of detection of lead by metallothionein can be due to its weakness as an inducer of metallothionein at transcriptional level (Kramer *et al.,* 1996; Kaji *et al.,* 1994).

Metallothionein and alpha tubulin have both qualitative and quantitative application potential for assessment of both heavy metals prevalence and duration in polluted *An. gambiae s.s.* larval habitat as well as adaptation/ resistance of the same to the pollutants. In this respect, alpha tubulin can effectively be applied to monitor prevalence of higher

cadmium presence and larval adaptation status in the habitats than metallothionein, which is more sensitive by detecting prevalence\adaptation following fewer selection generations. Thus alpha tubulin would ideally be used to evaluate both cadmium prevalence and mosquito adaptation where rapid urbanization is taking place since cadmium has general industrial origin. On the other hand, metallothionein is a good marker for detection of heavy metal prevalence and adaptation of *An. gambiae* to the metals in both new and old urban centers with cadmium emission. Metallothionein would also be applicable in agricultural areas where copper pollution is common from fertilizers. However, the efficiency of this molecular screening approach is critically dependent on bioavailabilty of the heavy metals of interest since in the environment. The heavy metals co-exist with other essential and heavy metals in various complexes that may affect their assimilation and sequestration. For example, cadmium uptake in rat hepatocytes is inhibited by copper, iron, zinc and mercury in a competitive manner, and the efflux is not inhibited by the metals except mercury thus affecting overall cadmium bioaccumulation (Blazka and Shaikh, 1992). This phenomenon is dependent on transport pathway, morphological and functional differences between the cell membranes (Shaikh *et al.*, 1995).

4.1.4 Differential Induction of Proteins in *An. gambiae ss* Larvae in Response to Selection by Heavy Metals

The findings demonstrate that adaptation of *An. gambiae s.s* larvae to increasing heavy metals challenge, is mediated by differential expression of several genes. The physiological responses appear to be both metal and concentration dependent, as

observed in *Aedes aegypti* larvae responses to cadmium and copper (Rayms-Keller *et al.*, 1998).

The low molecular weight proteins up regulated in response to heavy metals challenge may be metallothionein and mucin, which appear to play important role in the cellular defense system against heavy toxicity (Coogan *et al.*, 1994; Beyersmann and Hechtenberg, 1997; Rymes-Keller *et al.*, 2000), and biomineralization, as a strategy of counteracting increasing cellular levels of the heavy metals. Matallothionein coutract the heavy metal toxicity by binding to them hence reducing their bio-availability. Mucin lines the midgut of the insect and prevents xenobiotics, including heavy metals, from getting into the insect hemocoel. However, relative low expression in the fifth generation could be related to adaptation of the larvae to the heavy metals, representing those terminal genes counteracting heavy metals toxicity in the response cascade, such as metallothionein. However, the underlying mechanism mediating reduced expression of the low molecular weights protein concomitant with generations is not clear. Most differentially expressed genes that were neither acidic nor of low molecular weight did not display specific pattern across all metals and generations, a factor attributable to neoplastic transformation, transforming healthy somatic cells into a cancer cells, induced by non-cell-specific carcinogenic potential of metals such as cadmium (Terracio and Nachtigal, 1988). Similarity of both molecular weight and isoelectric point distribution patterns of both differentially and constitutively expressed protein spots is within expectation when overall effect of the heavy metals on gene expression is considered. For example, induction of cadmium specific genes are generally mediated thorough

interference with cellular signal transduction pathways, and mostly consist of constitutively expressed genes (Jin and Ringertz, 1990; Beyersmann and Hechtenberg, 1997).

Significant variation in pattern of magnitude of constitutively expressed spots among generations in some metals can conversely be related to induction of metal specific genes. Cadmium is a better inducer of metallothionein than lead (Goldstein, 1993) while copper is an essential element, relatively becoming toxic only at elevated levels (Hare, 1992). High potency of cadmium relative to copper and lead is demonstrated by the significant differences in expression between generations. The relative weakness of copper can be attributed to its assimilation as an essential element (Hare, 1992) and that of lead to its inherent relative weakness as an inducer of some heavy metal responsive genes (Goldstein, 1993; Kramer *et al.*, 1996; Kaji *et al.*, 1994). However, similarity in response in the first generation is attributable to 'broad spectrum' vanguard cellular gene induction to heavy metals challenge, prior to induction of heavy metals-type induction. Such general responses include immediate early genes (IEGs) (Matsuoka and Call, 1995) and glutathione (Singhal *et al.*, 1987; Chin and Templeton, 1993)

There were variations among controls between generations, reflecting generation to generation changes in the mosquito population sampled. However, the significant variations in induction and expression of the genes among metals between generations can be a reflection of the dynamics underlying gene expression in response to the heavy metals. This can be in a bid to counteract heavy metal toxicity by regulation of some

genes counteracting toxicity. This is underscored by observed cadmium chloride induction of some IEGs with differing time courses (Matsuoka and Call, 1995) and simultaneously enhance glutathione peroxidase and xanthine oxidase, and reduce glutathione reductase and catalase (Koizumi and Li (1992). Additionally, sub-lethal cadmium has potential to simultaneously inhibit protein synthesis and differentially increase synthesis of certain heat shock proteins (Ovelgonne *et al.*, 1995).

4.2 Conclusions

1 Concentrations of most heavy metals in aquatic larval habitats in urban Kisumu and Malindi are relatively higher than WHO recommended levels in drinking water with possible ecological consequences. Planning and drainage appears to influence the distribution of the heavy metals in Malindi. The metals appears to have a common origin in both cities. Although presence of *Ae. aegypti* and *An. gambiae* was influenced by concentrations of some heavy metals, most mosquitoes species, including some anophelines, do not appear to be influenced by the current concentrations of heavy metals, suggesting possible development of resistance of the population to the heavy metals in their habitats. The resistance to heavy metal has possible vector-borne diseases epidemiological implications since some vectors are supported by habitats that are customarily overlooked in environmental/habitat management programs in eradication of malaria mosquito vector.

2 *Anopheles gambiae* s.s. can rapidly develop resistance to heavy metals exposure. The resistance comes at significant biological costs that would reduce biological fitness of the resistant populations in non-polluted habitats in nature by adversely affecting all the developmental stages by reducing their capacity to grow and proliferate, relative to the naïve population. The reduced biological fitness may be attributed to both genetic and non-genetic factors.

3 Expressions of heavy metal responsive metallothionein and alpha tubulin genes are able to distinguish responses to different heavy metals and/or the selection pressure/duration in third instar *An. gambiae* larvae. This has the potential application in assessment of both heavy metals prevalence in polluted *An. gambiae s.s.* larval habitat and adaptation/ resistance status of local *An. gambiae* populations to heavy metal pollution. This in turn will provide insight on the potential contribution of polluted habitats to malaria epidemiology

4 Development of resistance in *An. gambiae s.s* to heavy metals challenge is rapid and appears to be mediated by differential expression of some specific heavy metals responsive genes. The magnitude and pattern of the differential responses of the genes may be related to inherent toxicity of each respective heavy metal. The characteristic response can empirically serve as a molecular marker for assessment of anopheline adaptation status to heavy metals pollution in their natural habitats. The magnitude and pattern of differential expressions can also be applied as molecular

markers to determine the magnitude and extent of environmental pollution through, anopheline larval habitats.

5 Overall, this study will facilitate designing of environmental management programs for mosquito control by lending insights into the potential contribution of heavy metal polluted larval habitats as sites where vectors proliferate. These sites are traditionally overlooked in such programs especially those targeting anophelines. Such program includes vector surveillance and application of biocides, larvicides, adulticides and drainage. The study also suggests that the *An. gambiae s.s* in more polluted environments e.g urban areas and in less polluted environments e.g rural environment may have different ecology and hence may warrant different approach in the control program.

4.3 Recommendations

1 Despite low level of industrial activities in urban Kisumu and Maliindi, the high levels of heavy metals presence in the larval habitats suggests that there should be an increased awareness for rational management of aquatic pollution, including control of waste discharges into environment.

2 The effect of heavy metals resistance in *An. gambiae ss* vector competence should be investigated since this will provide putative insight into the effect of adaptation of the mosquitoes to heavy metals pollution in nature on malaria epidemiology in polluted environments where the heavy metals resistant strain predominates..

3 Investigations should be conducted on several heavy metal responsive genes in other organisms and cell lines in *An. gambiae s.s* as functional homologues in order to establish molecular tools that can detect broad spectrum (several) heavy metals resistance/ adaptation in *An. gambiae* ss.

4 While heavy metal specific differentially expressed proteins were detected, the information obtained can further be complemented by characterization of the spots by mass spectrometry and establish putative function by analysis of the spots against *An. gambiae* protein database (Ribeiro *et al.*, 2004).

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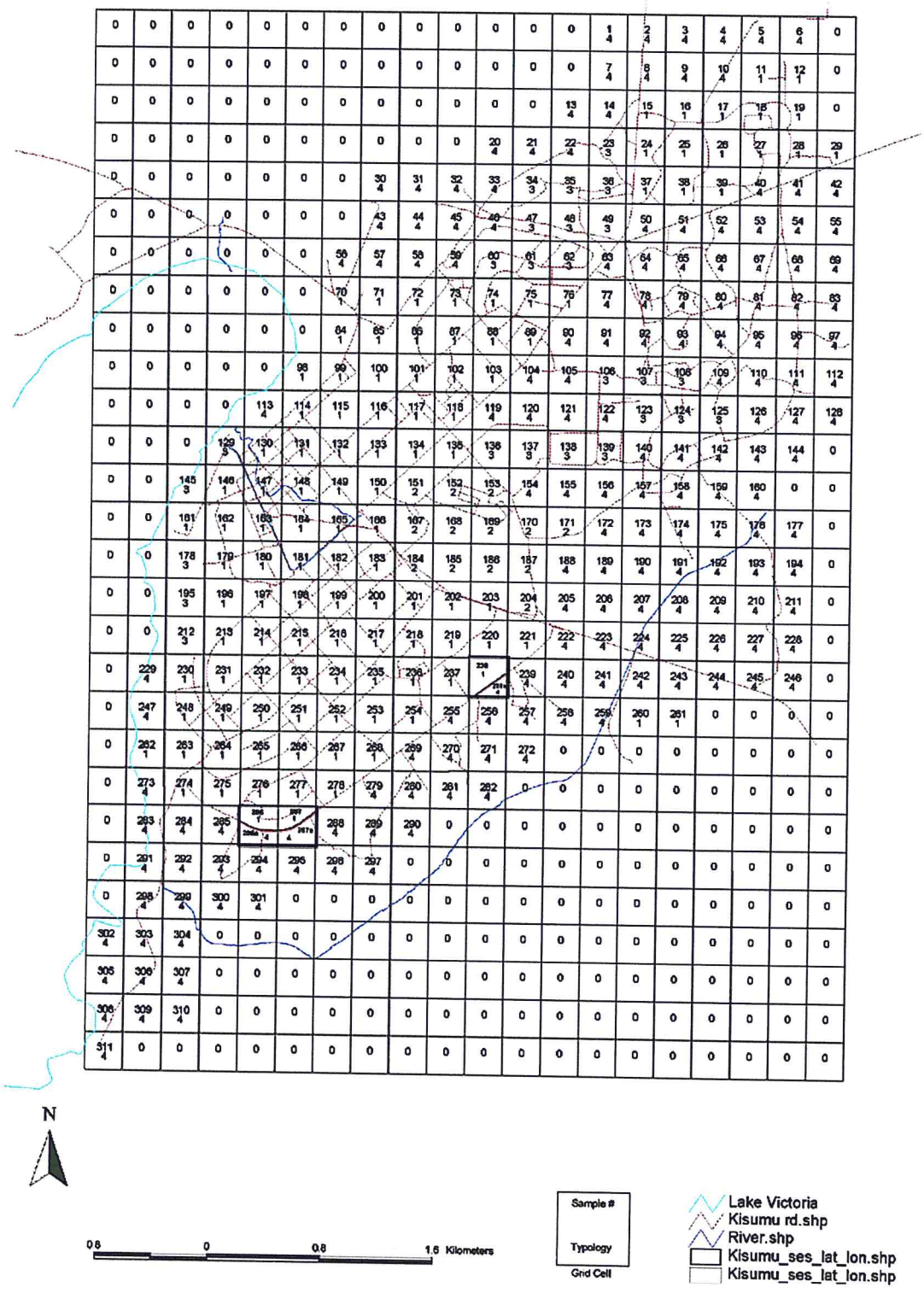
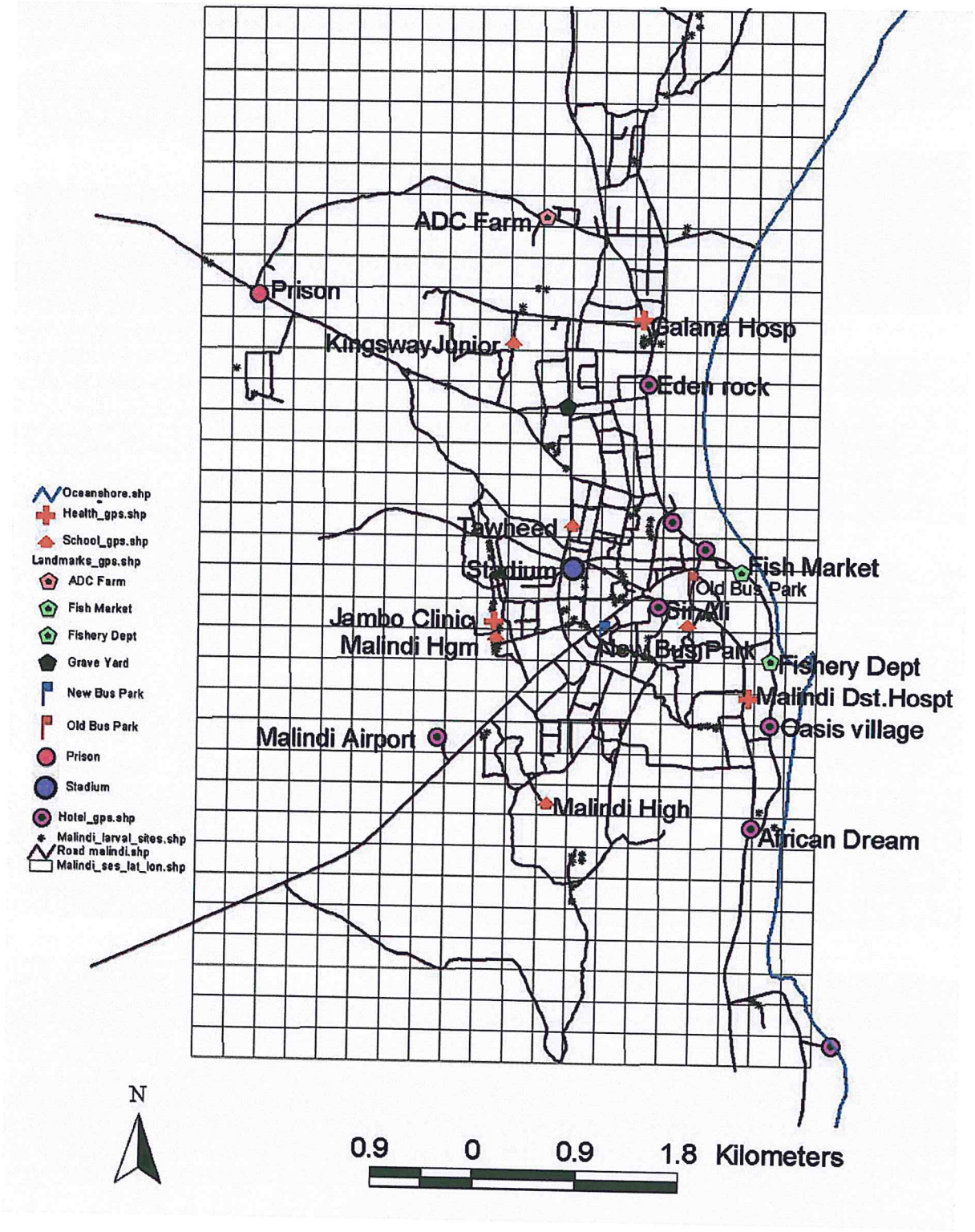
Appendices

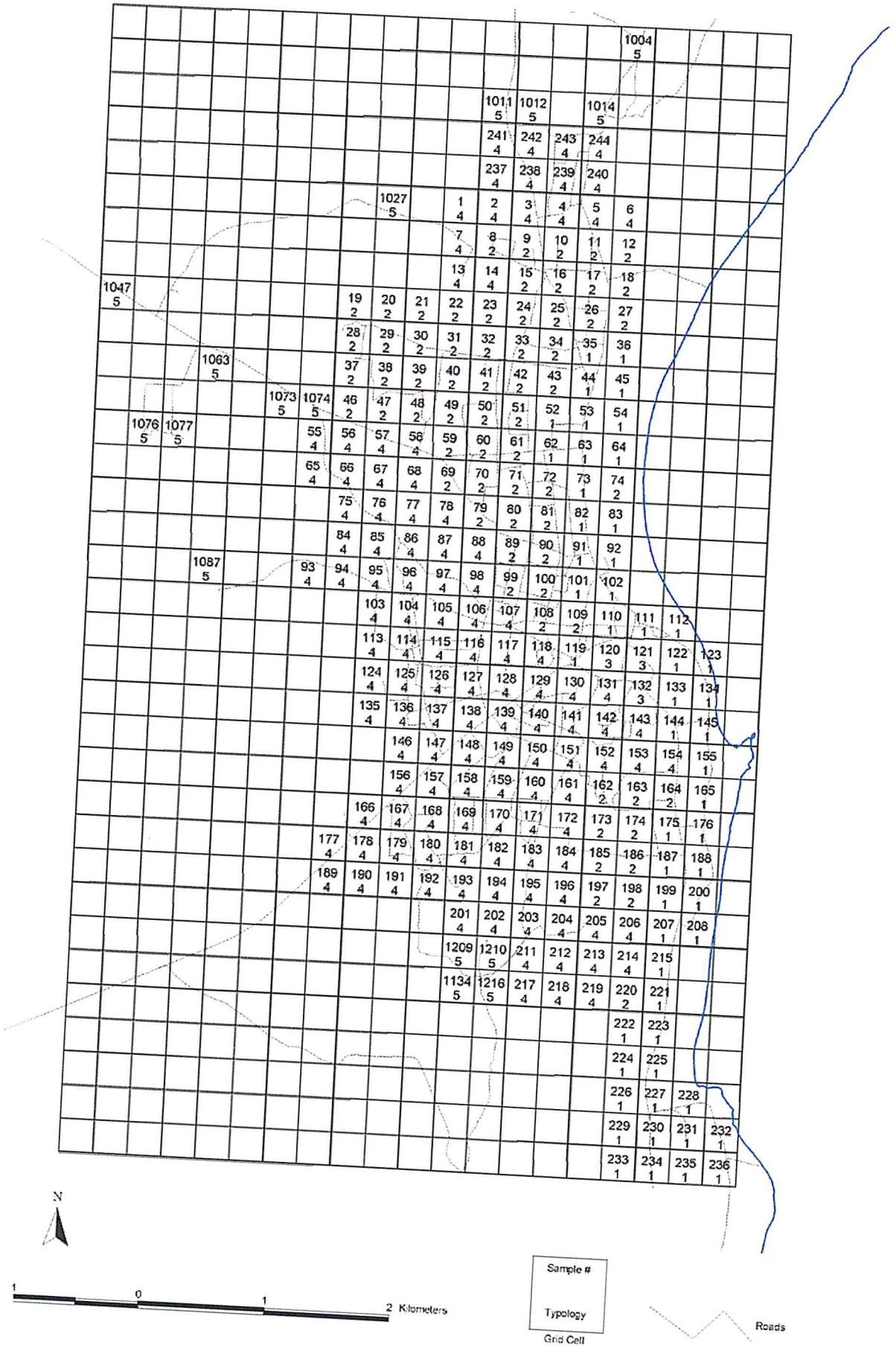
6.1. Kisumu Sampling Base Map

6.2. Kisumu Sampling Topology Map

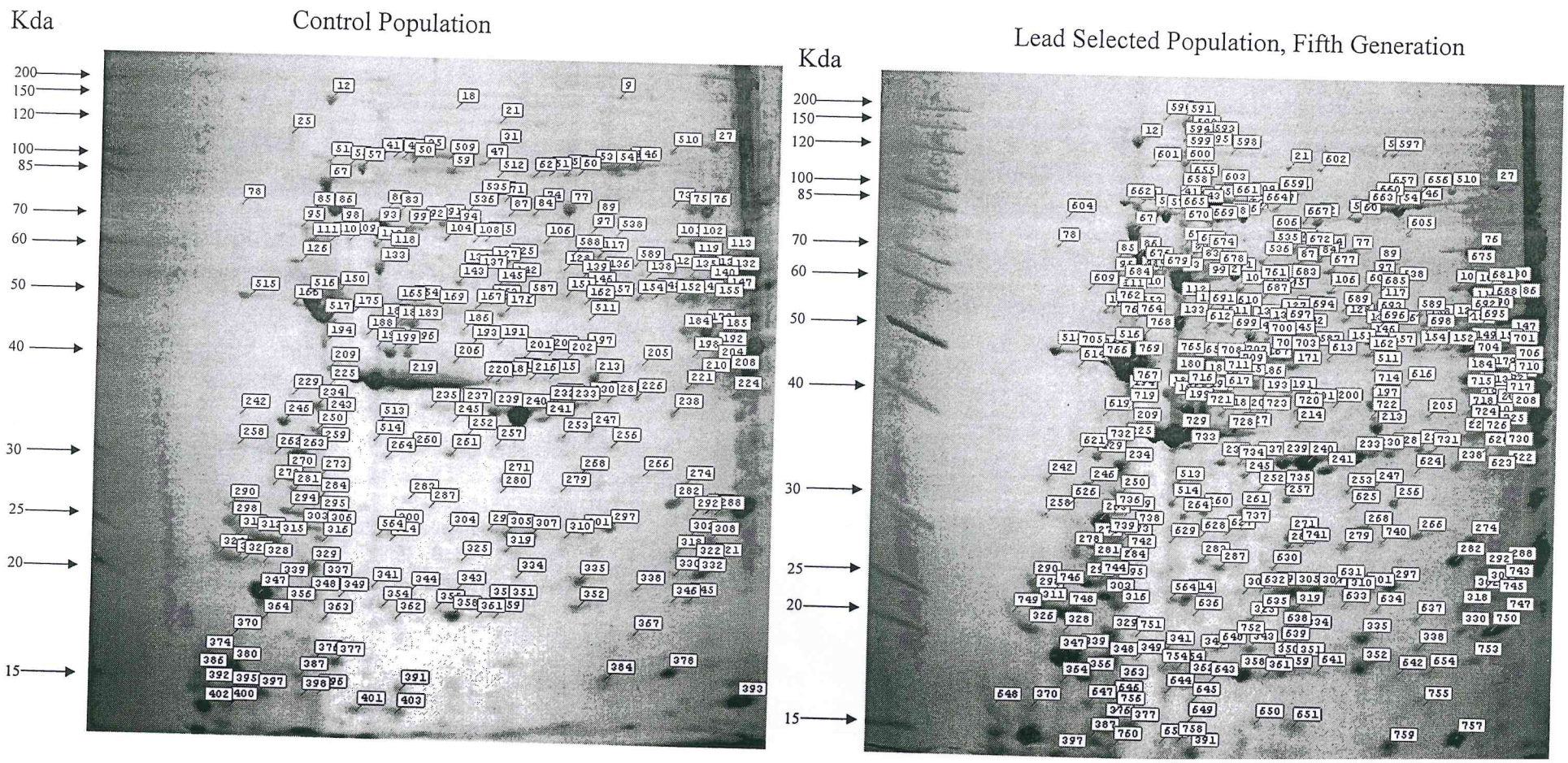


6.3 Malindi Sampling Base Map



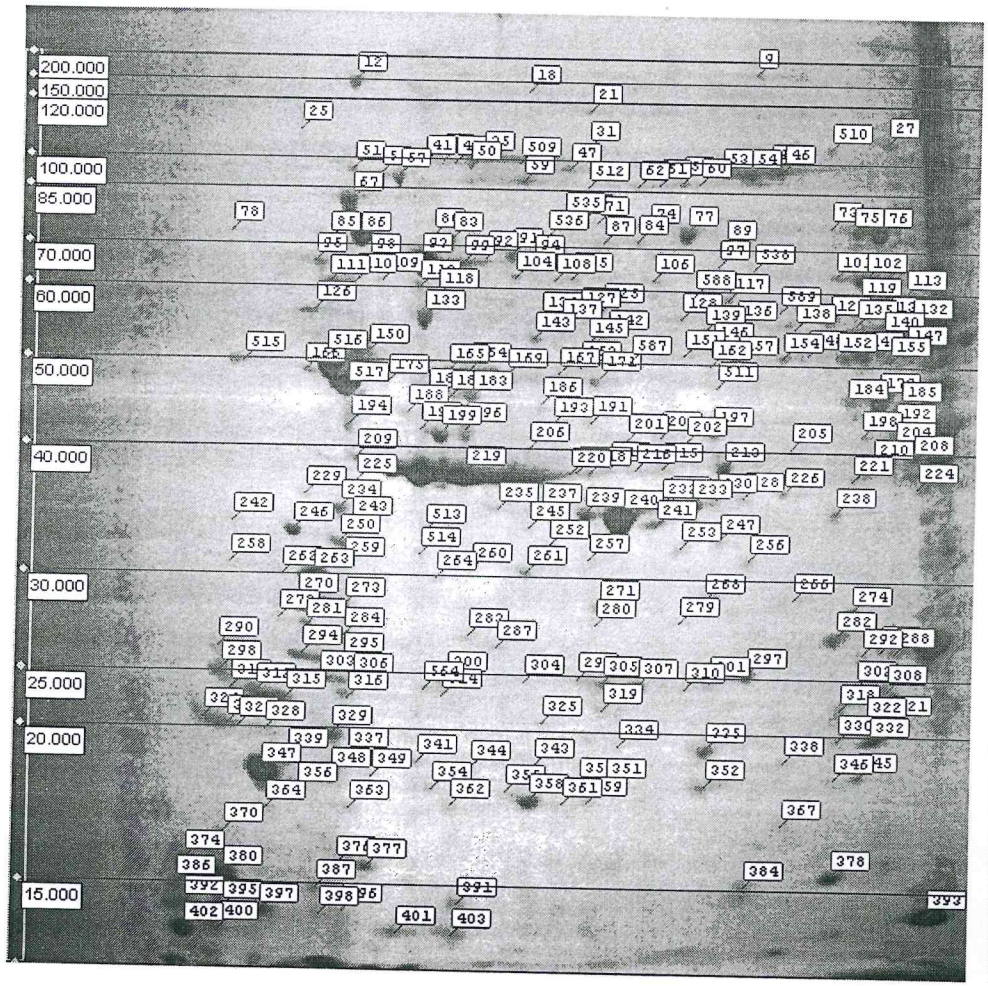

N
1 0 1 2 Kilometers
Sample #
Typology
Grid Cell
Roads

6.5. Identification of Expressed Protein Spots Spot in *Anopheles gambiae* Third Instar Larvae Control and Heavy Metal selected Populations (Example- Fifth Generation Lead Selection)

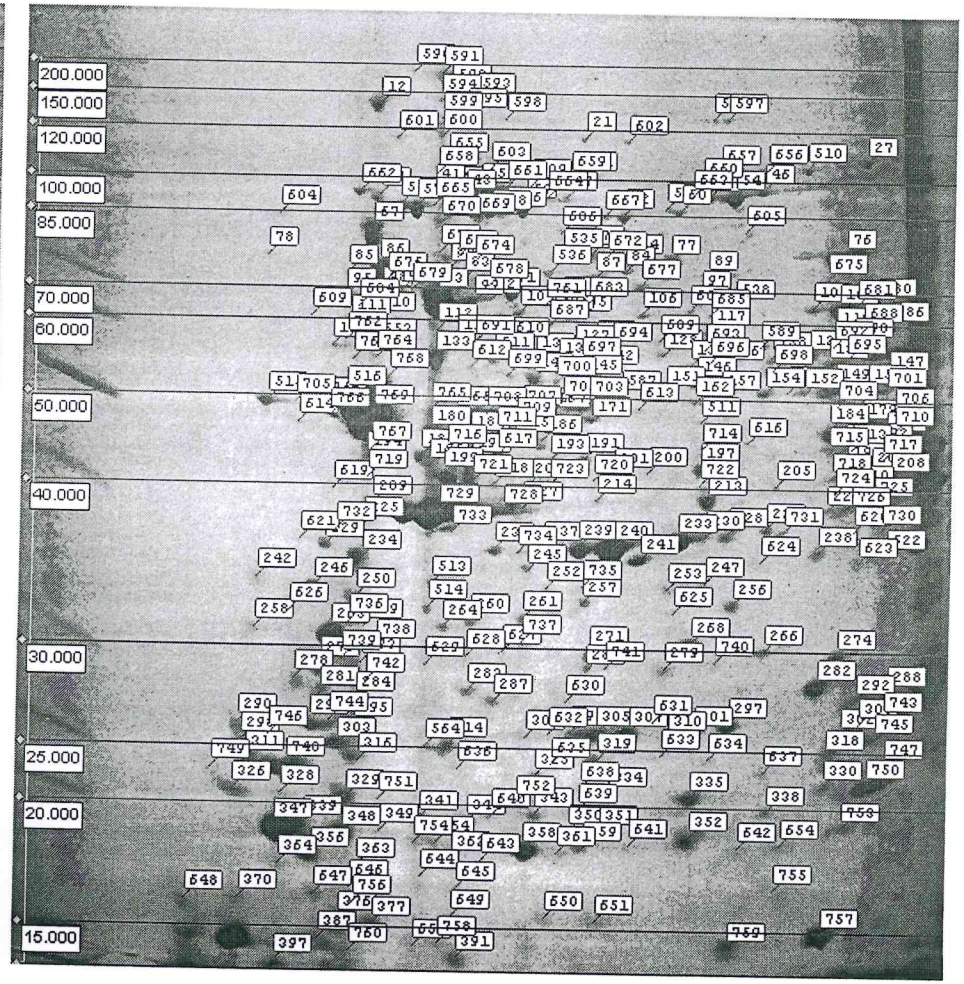


6.6. Calibration of Molecular Weights of Expressed Protein Spots Spot in *Anopheles gambiae* Third Instar Larvae Control and Heavy Metal selected Populations (Example- Fifth Generation Lead Selection)

Control Population

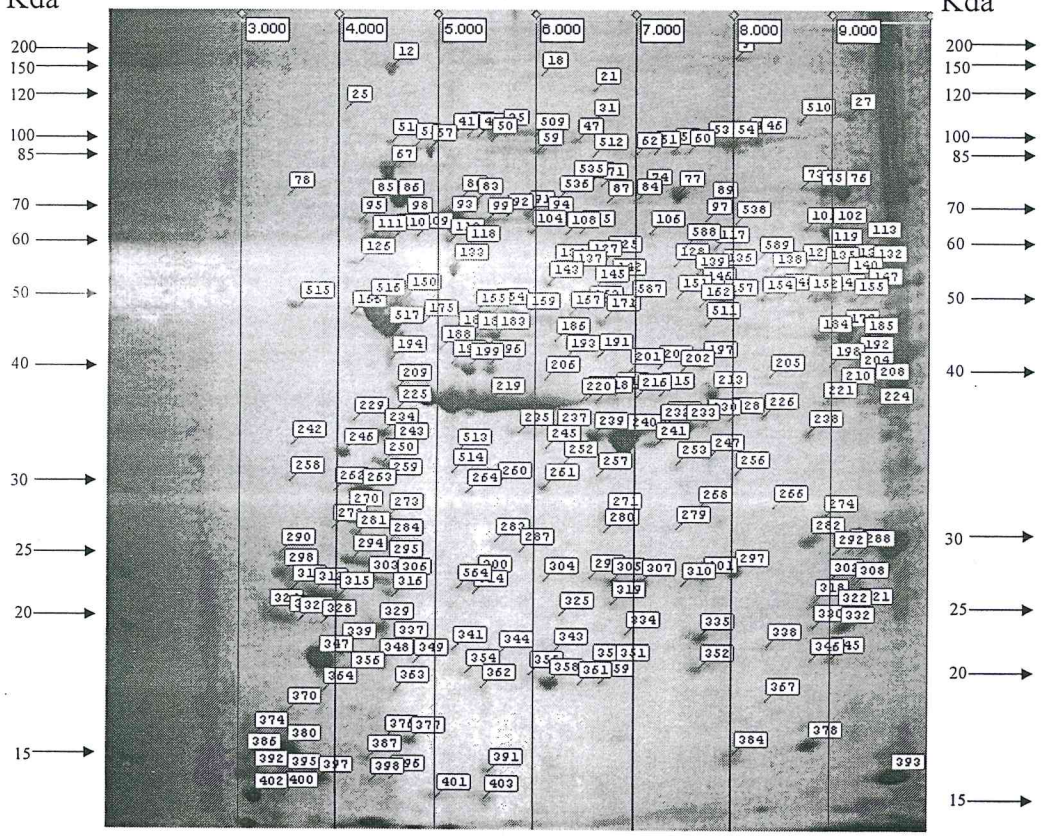


Lead Selected Population, Fifth Generation

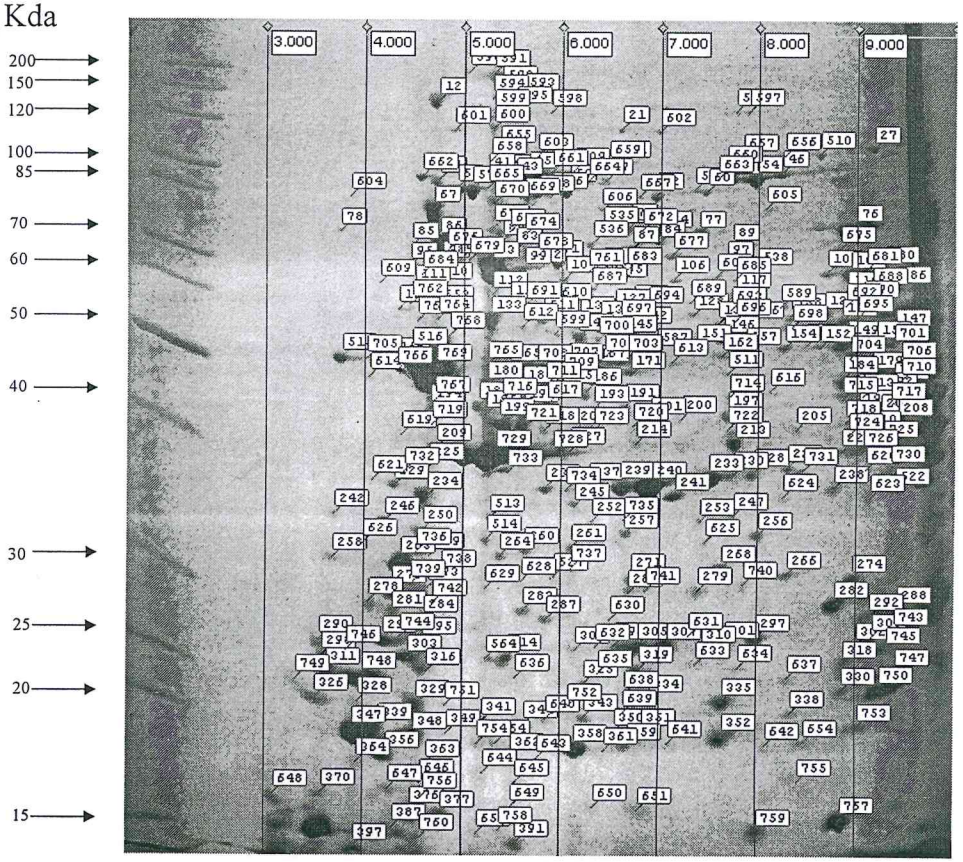


6.7. Calibration of Iso-electric Points (PI) of Expressed Protein Spots Spot in *Anopheles gambiae* Third Instar Larvae Control and Heavy Metal selected Populations (Example- Fifth Generation Lead Selection)

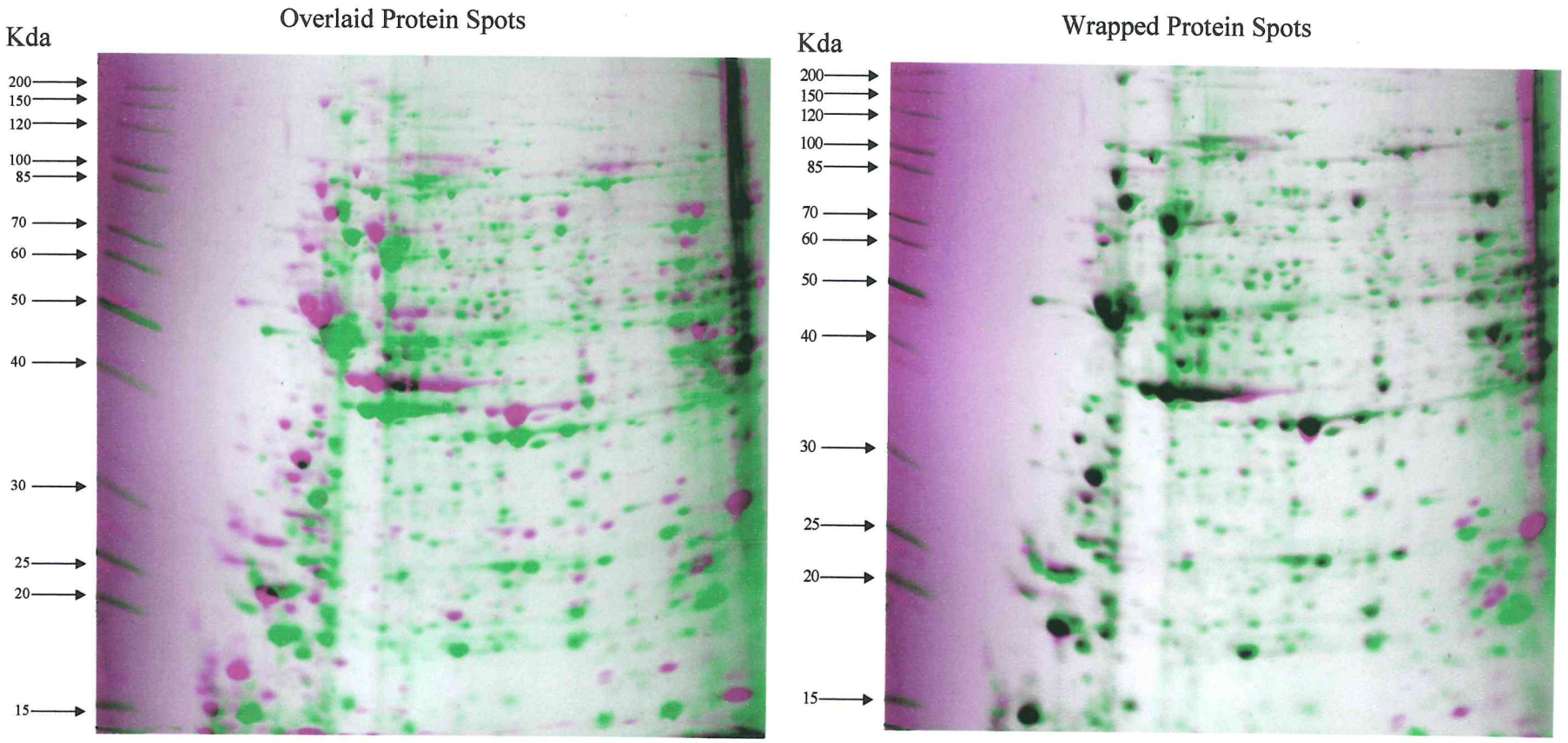
Control Population



Lead Selected Population, Fifth Generation



6.8. Expressed Protein Spots Spot in *Anopheles gambiae* Third Instar Larvae Control and Heavy Metal selected Populations Gels Overlaid and Wrapped to each other (Example- Fifth Generation Lead Selection)



Black spots = common, Pink spots= Non exposed (down regulated) and Green spots= Exposed (up regulated)