DETECTION OF BIOLOGICAL EFFECTS OF ENVIRONMENTAL POLLUTANTS OF THE KAFUE RIVER ON THE KAFUE LECHWE (*KOBUS LECHE KAFUENSIS*) BY CHARACTERIZATION OF SELECTED BIOMARKERS

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Clinical Pathology

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DECLARATION

I, Ethel M'kandawire do hereby declare that the contents of the thesis being submitted herein are my original work and they have not been previously submitted to any university for the award of a degree or any other qualification.

Signature…….

Date……

CERTIFICATE OF APPROVAL

This thesis submitted by Ethel M'kandawire, is approved as fulfilling the requirements for the award of the degree of Masters of Science in Veterinary Clinical Pathology at the University of Zambia.

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Chairman (Board of Examiners) Signature

ABSTRACT

A lot of research in ecotoxicology is currently focused on identifying and developing suitable biomarkers for use in assessing environmental pollution. Thus this study was aimed at assessing the potential of Cytochrome P450 1A1 (CYP 1A1), Cytochrome P450 3A (CYP 3A) and Metallothionein1 (MT 1) as biomarkers in the Kafue lechwe in response to pollutants in the Kafue river. Kafue lechwe liver samples from Lochnivar GMA (n=23) and Blue Lagoon GMA (n=15) were analyzed for the following biomarkers: CYP 1A1, CYP 3A and MT I. Complementary deoxyribonucleic acid (cDNA) was cloned and CYP lAl, CYP 3A and MT 1 partial nucleotide sequences were isolated from liver of Kafue lechwe. The deduced partial nucleotide sequence of lechwe CYP 1A1 revealed higher identities with that of sheep (98%) and cattle (97%). Lechwe CYP 3A revealed higher identities with that of CYP 3A24 of sheep (97%) and CYP 3A4 of cattle (95%). Lechwe MT 1 was closely related to MT of sheep (97%) and MT 1E of cattle (97%). In the phylogenetic analysis, the lechwe CYP 1A1 isoform was located beside sheep and cattle CYP 1A1. The lechwe CYP 3A isoform was located beside sheep CYP 3A24 and cattle CYP 3A4 and lechwe MT 1 isoform was clustered in the same group as that of sheep MT and cattle MT 1E. We then determined the mRNA expression levels of CYP 1A1, CYP 3A and MT 1 as biomarkers of pollution in liver of Kafue lechwe by relative quantitative real time reverse transcription polymerase chain reaction. The study showed that hepatic CYP 1A1, CYP 3A and MT 1 mRNA levels were expressed in all the lechwe samples from both Lochnivar and Blue Lagoon GMAs. However, there was a wide inter-individual variation in the gene expression of all the 3 genes in Kafue lechwe from both sites. Therefore a comparison of the gene expression levels between the two GMAs was determined by normalizing the relative quantities data for Blue Lagoon to one. Single factor ANOVA was employed for the statistical analysis. There was a 6 fold increase in CYP 1A1 mRNA expression in lechwe from Lochnivar GMA compared to lechwe from Blue Lagoon GMA and the difference was significant ($P<0.05$). There was no significant difference in CYP 3A and MT 1 mRNA expression levels between lechwe from Lochnivar GMA and lechwe from Blue Lagoon GMA ($P>0.05$). The study therefore demonstrates that hepatic CYP 1A1, CYP 3A and MT 1 mRNA expression levels in Kafue lechwe has the potential to be used as biomarkers of exposure and effect to pollution in the Kafue river.

DEDICATION

I dedicate this work to my father for his insistence to see me attain higher education and achieve better things. To my mother, I thank you for urging me to be self dependent and be a family asset. My sisters and brother for the encouragements they gave me to attain higher heights and to be a better person. To my husband, I owe the biggest debt of all. Thank you for all your patience in the months I struggled to balance life at home and completing my thesis.

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LIST OF ABBREVIATIONS

UNEP	United Nations Environment Program
UN	United Nations
DDT	Dichlorodiphenyltrichloroethane
GDP	Gross Domestic Product
FeS_2	Pyrite
$CuFeS_2$	Chalcopyrite
Cu_5FeS_4	Bornite
CuS_2	Chalcocite
$Cu_9 S_5$	Digenite
Co_3S_4	Linnaeite
$Cu(Co,Ni)_2S_4$	Carrolite
PCBs	Polychlorinated biphenyls
Cu	Copper
Pb	Lead
Fe	Iron
Co	Cobalt
Ni	Nickel
Zn	Zinc
NSE	Nakambala Sugar Estates
ECZ	Environmental Council of Zambia

NCZ	Nitrogen Chemicals of Zambia
NPK	Nitrogen, Phosphorous, and Potash
KSTP	Kafue Sewerage Treatment Plant
COD	Chemical Oxygen Demand
BOD	Biochemical Oxygen Demand
ZAWA	Zambia Wildlife Authority
WTO	World Trade Organization
WWF	World Wildlife Fund
JICA	Japan International Cooperation Agency
ZCCM	Zambia Consolidated Copper Mines
pp'-DDE	Dichlorodiphenyldichloroethene
pp'-DDD	1-1 dichloro-2,2 bis (p-chlorophenyl) ethane
PAHs	Polycyclic Aromatic Hydrocarbons
TCDDs	2, 3, 7, 8 tetrachlorodibenzo-p-dioxin
CYP 1A1	Cytochrome P450 1A1
CYP 3A	Cytochrome P450 3A
MT 1	Metallothionein 1
USEPA	United States Environmental Protection Agency
NRC	National Research Council
CYP 450	Cytochrome P450
MFO	Monooxygenase or Mixed-Function Oxygenase

NADPH	Nicotinamide adenine dinucleotide phosphate
Fe^{3+}	Ferric iron
Fe^{2+}	Ferrous iron
Cyt b5	Cytochrome b5
AhR	Aryl Hydrocarbon Receptor
CAR	Constitutive Androstane Receptor
PXR	Pregnane X Receptor
PPAR	Peroxisome Proliferator-activated Receptor
CYP 1A	Cytochrome P450 1A
CYP 1B	Cytochrome P450 1B
CYP 1C	Cytochrome P450 1C
CYP 2	Cytochrome P450 2
CYP 2B	Cytochrome P450 2B
CYP 2C	Cytochrome P450 2C
CYP 2D	Cytochrome P450 2D
CYP 2C9	Cytochrome P450 2C9
CYP 4	Cytochrome P450 4
CYP 3A65	Cytochrome P450 3A65
PCDD	Polychlorinated dibenzo-p-dioxin
PCDF	Polychlorinated Dibenzofurans
AhR	Aryl hydrocarbon Receptor

Arnt	AhR-nuclear translocator
DRE	Dioxin-Responsive Elements
XRE	Xenobiotic Responsive Elements
UDP-GT	Uridine 5-Diphosphate-Glucuronyl Transferase
GST	Glutathione- S-Transferases
OCs	Organochlorine Contaminants
PB-type	Phenobarbital type
RXR	Retinoid X Receptor
SXR	Steroid and Xenobiotic Receptor
XREM	Xenobiotic Responsive Enhancer Module
EROD	Ethoxyresorufin O-Deethylase
AHH	Aryl Hydrocarbon Hydroxylase
PCN	Polychlorinated Naphthalenes
BaP	Benzo(a)pyrene
cDNA	Complimentary Deoxyribonucleic Acid
ELISA	Enzyme-linked immunosorbent assay
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
MTs	Metallothioneins
ExPASy	Expert Protein Analysis System

Cd	Cadmium
Hg	Mercury
Ag	Silver
ROS	Reactive Oxygen Species
MRE's	Metal-Responsive Elements
MTF-1	Metal Transcription Factor-1
DNA	Deoxyribonucleic Acid
PKC	Protein Kinase C
PI3K	Phosphoinositol- 3 Kinase
JNK	c-Jun N-Terminal Kinase
GMAs	Game Management Areas
TRI	Total RNA Isolation
DEPC	Diethylpyrocarbonate
RT	Reverse Transcription/Transcriptase
DNTP	Deoxyribonucleotide Triphosphate
TBE	Tris Boric Acid EDTA
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
NJ	Neighbor Joining
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
SEM	Standard Error about the Mean

ANOVA	Analysis of Variance
TB	Tuberculosis
ORF	Open Reading Frame

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CHAPTER 1.0 INTRODUCTION

1.1 Global Perspective on Environmental Pollution

During the 20th century, an increasing consciousness of the harm that human development causes to the environment and the consequences to human health as well as loss of biodiversity became evident. In the 1970's, the environmental standards that would ensure a healthier planet were given due attention by international organizations. The Stockholm Conference on Human Environment in 1972 was a major step forward for the initiation of the International Environmental Action (United Nations Environment Program (UNEP), 1972). Efforts to reverse loss of environmental resources while ensuring sustainable economic growth and social development are also addressed in the United Nations Millennium Development Goals (United Nations (UN), 2006).

The world has seen a number of developmental transformations through advanced technology, industrialization, intensification and modernization of agriculture, urbanization and increased exploitation of natural resources. These have contributed greatly to the quantity and diversity of pollutants that are dispatched into soil, rivers and dams. The organic and inorganic pollutants entering water bodies that have resulted from anthropogenic activities disperse into water, sediment and biota compartments with devastating consequences. Adams (2001), attributed the major types of anthropogenic activities and their related stressors to agriculture (pesticides, herbicides, nutrients), pulp and paper (dioxins, resin acids, chlorophenolics), petroleum industry (polycyclic aromatic halogens, heavy metals), mining (heavy metals, suspended sediments, polycyclic aromatic hydrocarbons), domestic sewage (chlorine, nutrients, detergents) and power plants (temperature increases, chlorine). The effects of these pollutants once they reach water bodies include; effect on plants, humans, animals, global effects and technological hazards.

Pollutant stress in animals may be manifested in the impairment of physiological functions such as respiratory osmoregulation and energy metabolism or may appear as decreased reproductive efficiency, increased susceptibility to disease or predation and/or a decreased adaptability to the environment, anemia, eventual development of cancerous tissue growth (particularly in fish), neurological damage, and birth defects in offspring. The most obvious effect of environmental pollution on animals is death that occurs immediately after

exposure to high concentrations of toxic components. Animals that recover often suffer from a multitude of symptoms due to the inundation of their internal organs with toxic chemicals. In some cases animal populations such as wildlife have suffered severe losses or even faced extinction due to pollution. For example, the Bald Eagle (*Haliaeetus leucocephalus*), Peregrine Falcon (*Falco peregrinus*), and Brown Pelican (*Pelecanus occidentalis*) all nearly became extinct before it was discovered that the synthetic chemical, Dichlorodiphenyltrichloroethane (DDT) was the cause of devastating reproductive failure in these species (Anderson and Hickey, 1972).

1.2 The Zambian Perspective

Zambia has not been spared of human activities that often result in discharge of pollutants into rivers and one such river is the Kafue river that has over the years, acted as the drainage for both industrial and agricultural waste. There has been a direct and continuous input of pollutants into the Kafue river and a study of the effects of effluents on the Kafue river ecosystem has a high ecological relevance because the river is the source of water for humans, domestic and wild animals, and irrigation and for recreation activities. The river originates from the north western part of Zambia on the border to the Democratic Republic of Congo and flows through three provinces, namely the Copperbelt, the Central and the Southern Province, before its confluence with the Zambezi river (Figure 1). In the upstream the river flows in a southerly direction through the extensive mining area of the Copperbelt before passing through the Itezi-tezhi dam. Downstream, the river runs towards the east through the Kafue Flats, passes Kafue Gorge dam and then approaches its confluence with the Zambezi river. The total length of the Kafue river is approximately 1200 km and the catchment area is estimated at 157 000 km^2 at the confluence with Zambezi river, accounting for about 20 % of the total land area in Zambia. This makes Kafue river the largest tributary to the Zambezi river.

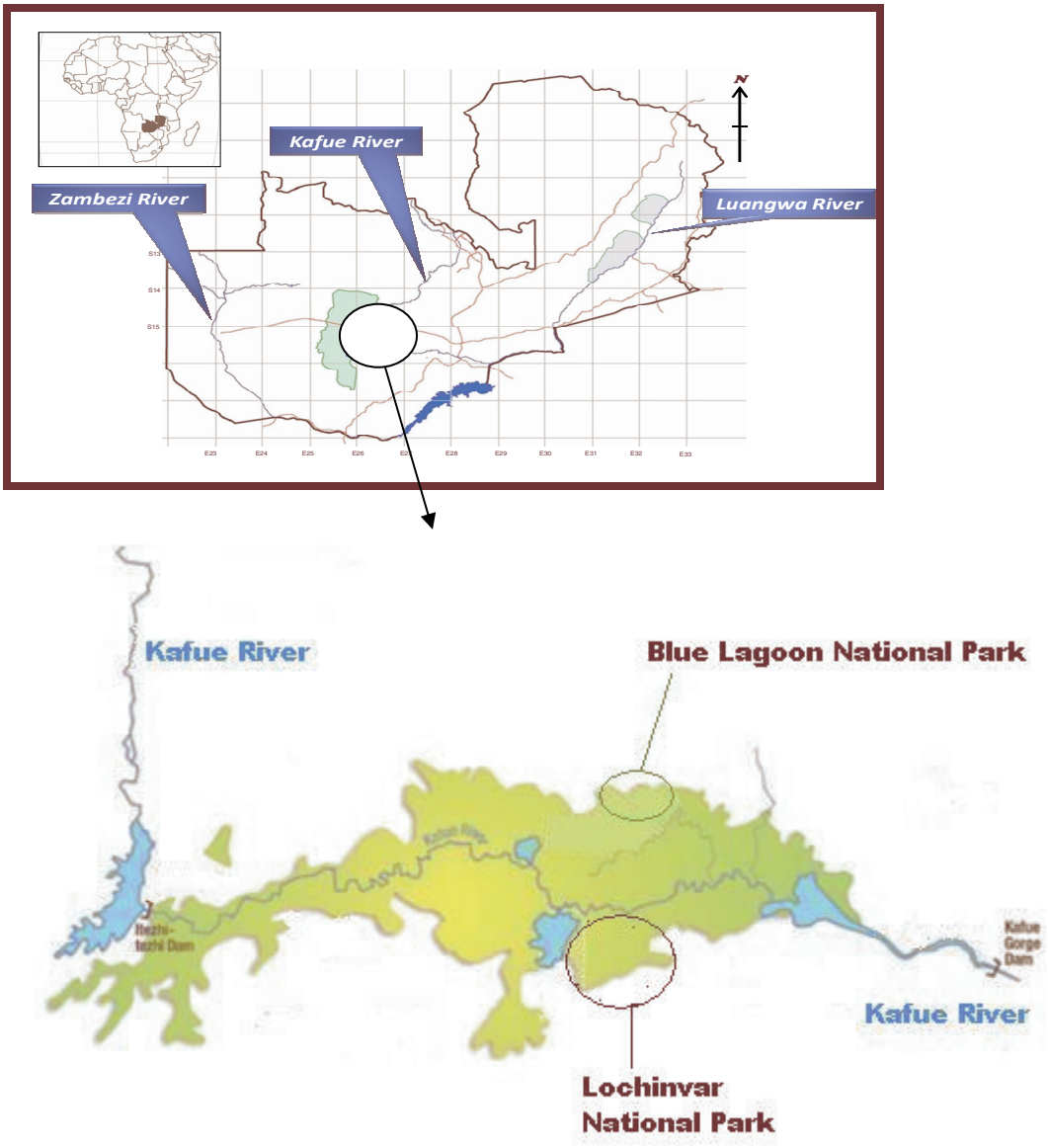

Figure 1: Maps showing the location of the Kafue river and Lochnivar and Blue Lagoon National Parks along the river

1.2.1 The Sources of Pollution to Kafue River

Point and non point sources of pollution of the Kafue river exist. Point sources emit effluents directly into water bodies and non-point sources take place indirectly from non specific or undefined sources in sufficient quantities to contribute to the pollution problem. Point sources are more recognized and responded to, compared to non point sources, whose contribution is often neglected despite its high input to the pollution load. Run off from agriculture are considered as the most important non-point sources of pollution of the Kafue river (Alsterhag and Petersson, 2004). There are many farms along the Kafue river which use a lot of agrochemicals including pesticides and herbicides which drain into the Kafue river. Point sources of the Kafue river include the following: Copperbelt mining areas, the Kafue basin with a lot of agricultural activities that include Mazabuka's sugar plantation (Nakambala Sugar Estates), Kafue town which has some industrial activity chiefly from a textile plant (African Textiles), chemical plant (Nitrogen Chemicals of Zambia), a leather processing industry (Bata Tannery) and a sewerage treatment plant (Kafue Sewerage Treatment Plant).

a) The Copperbelt Mining Areas

Mining is a familiar sight on the Zambian Copperbelt and has been for centuries (ZZAM, 1998). Commercial mining on the Copperbelt began in the1920s, and since then the region has been characterized as one of the highest densities of large-scale mines in the world. The area hosts one of the world's highest-grade copper and cobalt ore, and is estimated to contain 34 % and 10 % of global cobalt and copper reserves respectively (MBendi, 2000). The mining industry dominates the national economy, accounting for more than 60 % of Zambia's Gross Domestic Product (GDP) and 80 – 90 % of foreign revenue (Sinkala, 2001; ZZAM, 1998). Mineralization within the Copperbelt is characterized by pyrite (FeS_2), chalcopyrite ($CuFeS_2$), bornite (Cu_5FeS_4), chalcocite (CuS_2) digenite ($Cu_9\ S_5$), linnaeite (Co_3S_4) and carrolite ($Cu(Co,Ni)_2S_4$), embedded in carbonate-rich shales and argillite (Mendelsohn, 1961). The mines within the Copperbelt are localized along the

Kafue anticline, which is the dominant structural feature of the Copperbelt. Most streams within the area rise in grassy treeless areas occupying hollow saucer-shaped depressions, locally known as dambos. They are wet or swampy during the rainy season but may dry out during the dry season (Mendelsohn, 1961). Streams and rivers locally follow deeply weathered carbonate formations, and in gentle depressions of the late tertiary peneplain they cut open v-shaped notches (Mendelsohn, 1961). Within the urban part of the Copperbelt the Kafue river follows the Kafue anticline, near the site of present mining activities. Although the wealth and economic growth generated from the exploitation of the Copperbelt's mineral resources are crucial to the nation's growth and development (Kangwa, 2002), mining has long been considered one of the world's most environmentally degrading enterprises (Hoover and Hoover, 1950; Kelly, 1988; Marcus, 1997). Besides the direct impact on the landscape, mining and related industries on the copperbelt have produced large volumes of inorganic effluent especially tailings and slag into the Kafue river, which have been associated with various levels of environmental impact and degradation. The mines are also sources of polychlorinated biphenyls (PCBs). The river is also continuously being polluted with heavy metals such as copper (Cu), lead (Pb), iron (Fe), cobalt (Co), nickel (Ni) and zinc (Zn) from metal processing plants. Hence, the Zambian Copperbelt is burdened by an economic and environmental paradox. On the one hand, development and growth of the mining industry is crucial in addressing Zambia's social and economic plight. On the other hand, all natural drainage routes of mine effluent enter the dambo wetlands before discharging into larger waterways and ultimately the Kafue river (Limpitlaw, 2002).

b) Nakambala Sugar Estates (NSE)

The commercial sugar cane plantation and sugar processing of Nakambala Sugar Estates (NSE) in Mazabuka was established in 1964 and today it covers an area of about 12, 000 hectares. This makes NSE the main sugar producer in Zambia with a production large enough to export to neighboring countries after supplying the Zambian market. Fertilizer use is of importance at the sugar plantation and the fertilizers applied in the fields are diammonium phosphate, urea diammonium phosphate and murate potash. About 6, 400 tonnes of fertilizers are used annually (Environmental Council of Zambia (ECZ), 1999). It

is assumed that there are no major losses of fertilizer to the aquatic environment since the drainage water from the fields is recycled. However the biggest impact on the Kafue river from NSE is believed to originate from the factory, which uses more than 300 tonnes phosphorus in the purification process of the sugar cane. There are three discharge points from the purification factory into the aquatic environment: 26 East, 26 West and Simbotwe. 26 East and 26 West drain the eastern and the western parts of the estates respectively. The wastewater from the processing factory passes through effluent ponds and filter reed beds before it reaches the stream of Simbotwe, which also receives drainage water from some surrounding fields. The three discharges enter Kaleya river which is a tributary to the Kafue river.

c) Nitrogen Chemicals of Zambia (NCZ)

Nitrogen Chemicals of Zambia produced Ammonium nitrate, Nitrogen, Phosphorous, and Potash (Potassium) fertilizers (NPK-fertilizers) and sulphuric acid starting from 1970 up until the 1990's when it closed due to financial problems. During the last few years NCZ has had sporadic production and the NPK-fertilizer production is usually in operation. The NPK plant only produces gaseous waste and does not discharge any liquid effluents. When the other productions (Ammonium nitrate) are running their wastewaters are pre-treated and then pumped to manmade lagoons for further treatment before being discharged into the Kafue river. However, water is pumped from the pipe connecting NCZ with the lagoons, and from the lagoons themselves, to irrigate illegal farms in the area. As a result not fully treated wastewater reaches the river. Storm water from the area is collected in drains and joins the Kasenje stream, a tributary of the Kafue river (Alsterhag and Petersson, 2004).

d) Kafue Sewerage Treatment Plant (KSTP)

Kafue Sewerage Treatment Plant (KSTP) was constructed in 1972 and is based on biological treatment. The wastewater undergoes primary and secondary treatment and a succession of four lagoons before it finally flows into the Kafue river through a well-defined discharge canal. The estimated retention time through the full system is four

months. However, there are several small-scale farmers in the area who drain water from the system and thus interrupting the cleaning process of the wastewater. Roughly 15 % of the town's residential areas are connected to the sewerage treatment plant while the rest use pit latrines and septic tanks. Normally the wastewaters from industries that lack own facilities for purification is treated at Kafue Sewerage Treatment Plant. However, there are times when pipes connecting these industries with KSTP have been vandalized resulting in untreated wastewater being discharged into the Kafue river (Alsterhag and Petersson, 2004).

e) Kafue industrial area

The small stream of Kasenje runs through Kafue industrial area where among others Bata Tannery, Lee Yeast, Kafue Chemicals and African Textiles are located. Kasenje stream enters Chirumba Lagoon which is a part of the Kafue river upstream of the Kafue Railway Bridge. Bata Tannery uses various chemicals in tanning animal skins. Amongst these chemicals is chromium sulfate, which can easily be converted to either hexavalent or trivalent chromium. The effect of these chemicals on human and aquatic life is potentially lethal. Equally, the yeast production from Lee Yeast results in high concentrations of both chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in the wastewater. The net effect is the reduction in the river system's oxygen concentration, leading to toxic anaerobic conditions (Alsterhag and Petersson, 2004).

1.2.2 Dependants of the Kafue River affected by the Pollution

The dependants of the Kafue river are many (Sheppe, 1985) and include;

a) Wild mammals

Wild mammals dependent on the Kafue river are many and include buffaloes, zebras and antelopes. Most importantly, the Kafue river and its habitats provide for extensive endemism of rare mammals such as the Sitatunga (*Tragelaphus spekei*)

and Kafue lechwe (*Kobus leche kafuensis*). The Kafue lechwe as one of the dependents of the Kafue river and relevant to this study is discussed.

Kafue lechwe (*Kobus leche kafuensis*)

It is a medium-sized, semi-aquatic antelope that is endemic and is a predominant wildlife species of the Kafue river basin of Zambia (Sheppe, 1985). The lechwe antelope is confined to a relatively small area in the basin, particularly in and around Lochinvar and Blue Lagoon National Parks. They live in large groups that become further concentrated during the rainy season and the early dry season (November to June) when the range is flooded (Gallagher *et al*, 1972). The antelopes spend much of their time in water up to 50 cm in depth (Rottcher, 1976). They graze the grass (*Echinocola scabra, Sacciolepis africana, Termitaria spp*) growing in the shallow waters along the river front and are rarely seen far from the waterfront.

The lechwe occupies an important role in the food chain in the Kafue river basin. It is consumed by humans, vultures and predators and it is a major source of manure food for fish; the fish in turn are the major food for aquatic birds and humans (Figure 2).

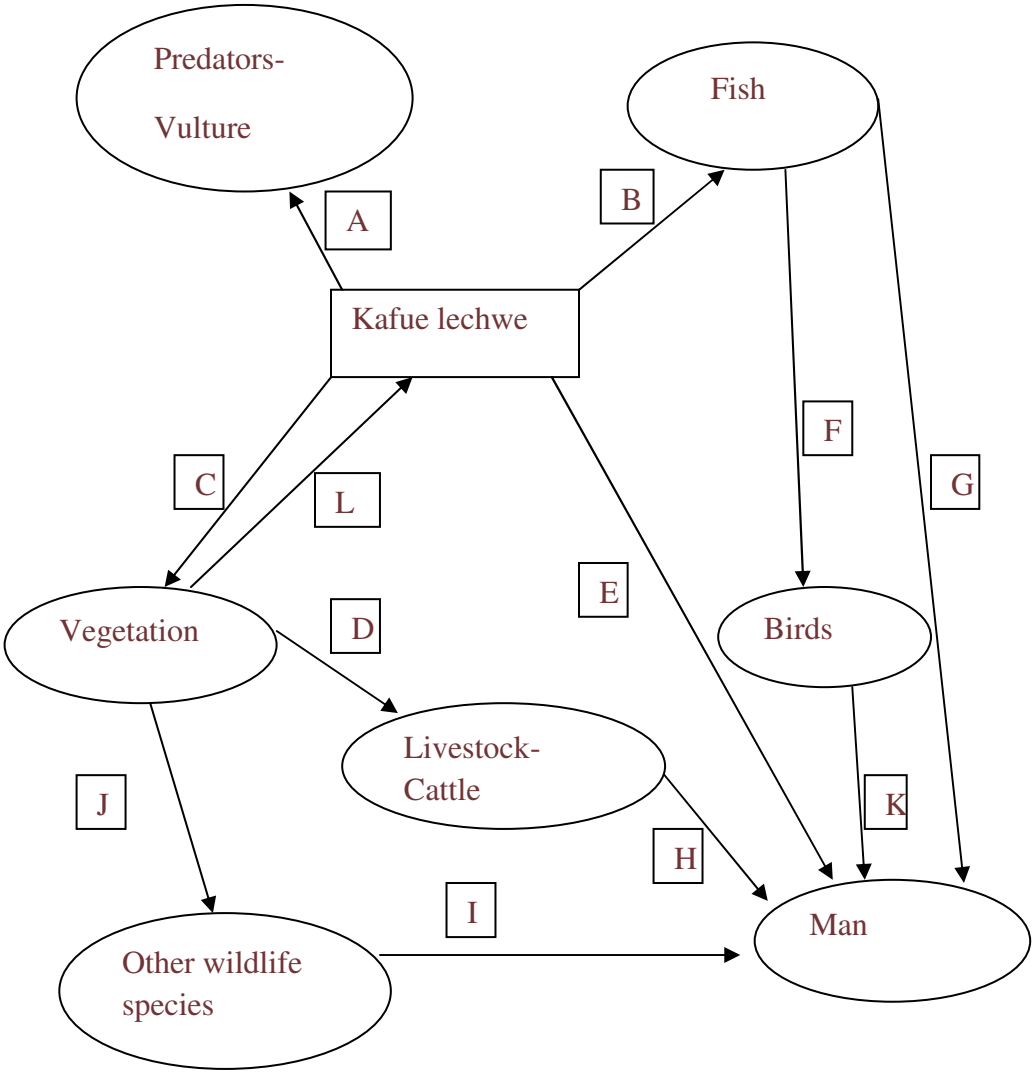

Figure 2: Ecological role of the Kafue lechwe on the Kafue Flats (Modified from Siamudaala *et al*, 2003)

Key:

A) Kafue lechwe carcasses available in the field
B) Manure from Lechwe
C) Manure fertilizing the pasture
D) Pasture for livestock
E) Source of protein and income for people
F) Food for aquatic birds
G) Source of protein and income
H) Source of protein and income
I) Source of protein and income
J) Pasture for other wildlife
J) Source of protein and income for people
K) Pasture for Kafue lechwe

The lechwe is one of the most highly hunted wild animals in Zambia for human food and its health status has significant economic and public health implications (Siamudaala *et al*, 2003). Between 1995 and 1999, a total of 4,679 lechwe carcasses were legally harvested according to official figures (Siamudaala *et al*, 2003). Of the total, 4,353 (93%) lechwe were hunted for game meat and 326 (7%) were taken on safari hunts, giving an average annual harvesting rate of 936 animals (Table 1). The average quantity of lechwe meat produced annually is estimated at 47.7 tons. The annual average for the period 1995 and 1999 of the number of people who consumed the meat was about 39,780. These figures reflect the official data of Zambia Wildlife Authority (ZAWA); the actual numbers of carcasses consumed and people who eat Kafue lechwe meat in Zambia are likely to be higher. The main consumption centers for both legal and illegal lechwe meat are Lusaka Mumbwa, Monze, Kafue, Namwala, Mazabuka, and Itezhi-tezhi districts (Figure 3). The revenue generated annually from national (citizen) and safari hunting is US $47,459 and US $60,315, respectively. The average income generated per animal from national and safari hunting during the said period was US $55 and US $925, respectively (Table 2).

Table 1: Number of Kafue lechwe hunted on the official quota between 1995 and 1999 (Siamudaala *et al*, 2003).

Year	No. animals hunted			No. animals hunted as % of total carcasses		Meat production and consumption	
	National hunting	Safari hunting	Total	National hunting	Safari hunting	Meat production in kg (tons)[a]	No. people consuming the meat[b]
1995	1,363	74	1,437	94.9	5.1	73,287 (73.3)	50,894
1996	457	66	523	87.4	12.6	26,673 (26.7)	22,228
1997	1,040	75	1,115	93.3	6.7	56,865 (56.9)	47,388
1998	668	54	722	92.5	7.5	36,822 (36.8)	30,685
1999	825	57	882	93.5	6.5	44,982 (45.0)	37,485
Total	4,353	326	4,679	93.0	7.0	238,629 (238.6)	198,858
Annual Average	871	65	936	93.1	6.9	47,736 (47.7)	39,780

[a] Mean dressing weight of 51.0 kg (Stafford *et al*, 1992)

[b] Annual average consumption of game meat per person of 1.2 kg (Chardonnet *et al*, 2002)

Figure 3: Kafue Flats Game Management Area, and Lochinvar and Blue Lagoon National Parks (Sheppe, 1985)

Table 2: Official data from ZAWA on the economic value of the utilization of the Kafue lechwe between 1995 and 1999 (Siamudaala *et al*, 2003)

Year	National hunting			Safari hunting		
	No. animals hunted	**Income, ZMK (US$)**	**Average income/animal, ZMK (US$)**	**No. animals hunted**	**Income, US$**	**Average income/ animal, US$**
1995	1,363	64,950,000 (72,858)	47,652 (53)	74	56,625	765
1996	457	52,900,000 (43,908)	115,755 (96)	66	62,700	950
1997	1,040	78,037,500 (58,936)	75,036 (57)	75	71,250	950
1998	668	60,120,000 (31,319)	90,000 (47)	54	54,000	1,000
1999	825	74,250,000 (30,275)	90,000 (37)	57	57,000	1,000
Total	4,353	330,257,500 (237,296)	75,869 (55)	326	301,575	925
Average per year	871	(47,459)	(58)	65	60,315	932

The lechwe is therefore of serious economical, ecological and conservation importance. It thus becomes imperative that continuous monitoring of pollutants of the Kafue river is made routine in the quest to remedy the effects of environmental pollution in animals that depend on the Kafue river.

b) Birds

The river and its habitats are home to over 400 species of birds including about 125 waterbirds. It hosts the world's largest population of wattle cranes (*Grus carunculatus*). Other notable birds are a large variety of ducks (*Anas spp*), geese (*Branta spp*), herons, egrets, shorebirds, pelicans, storks, ibises, and cranes. Others are fish eagles (*Haliaeetus vocifer*), darters (*Anhinga rufa*), and jacanas (*Actophilornis africanus*).

c) Fish

Fish species in the area include cichlids (*Sartherodon spp*), barbells (*Clarias spp*) and *Tilapia* spp, especially *T. niloticus.*

1.2.3 Zambian economic, social and environmental goods and services associated with the Kafue River

The river is the dominant source of water and food to various urban and rural settlements and enterprises. According to Mutale and Mondoka (1996), the river is the principal water source to all of the country's major towns, besides Livingstone. As such, the Kafue river's importance cannot be overstated as it supplies water to over 40% of Zambia's population, many of who are additionally dependent on its resources for other purposes (Table 3). Fifty five (55) species of Cichlid fish have been described in its waters, of which 23 are commercially important (Maar *et al*, 1966) and over 10 endemic species of fish (Sheppe, 1985). Communities rely on these fish stocks for over 70% of their daily protein intake and as the dominant source of revenue (Choongo, 1994). In addition, the Kafue river and its habitats provide for extensive endemism and biological diversity. The river and its habitats are home to over 400 species of birds, rare mammals and unique floodplain, riparian and wetland vegetation (Sheppe, 1985). As a result, the Kafue ecosystem constitutes a unique environment of social, economic and environmental importance, and international acclaim and recognition (Chooye and Drijver, 1995; RAMSAR, 2002; World Trade Organization (WTO), 1999; World Wildlife Fund (WWF), 2001).

Table 3: Zambian economic, social and environmental goods and services associated with the Kafue River adapted from Von der Heyden and New (2002d)

Sector	Examples	Town/ region
Agriculture	• maize, soya, cotton, wheat, groundnuts, sweet-potatoes, coffee, sugar cane and livestock	• Kitwe, Mazabuka, Mpongwe, Mkushi. Subsistence agriculture along the entire river
Industrial	• Mining, fertilizer and manufacturing	• Chingola, Kitwe, Kafue, Lusaka
Energy	Hydroelectric schemes • Itezhi-tezhi dam, Kafue Gorge Dam	• Southern Province
Domestic water	• Supply and security	• All major towns and large rural settlements along the entire river.
Game and nature reserves and hunting concessions	• Kafue National Park, Blue Lagoon National Park, Lochinvar National Park, Kafue Flats Game Management Areas	• Central and Southern Province
International heritage sites	• Kafue Flats, Lukanga Swamps	• Central and Southern Province

1.2.4 Status of Detection of Pollutants in the Kafue River and Environmental Impact on Animals Dependent on the River

Over the years a number of changes have occurred in fauna and flora diversity and community composition in the Kafue river, indicating a shift in the system's ecological balance (Sheppe, 1985). Various causes of this shift have been described, but none conclusively verified. Some authors (e.g. Mwase 1994; Norrgren *et al*, 2000) acknowledge the role of heavy metal pollutants from the Copperbelt mining area in the environmental degradation of the Kafue river ecosystem. Mining related effluent has entered the waterways of the Copperbelt for the past 70 years, resulting in extensive environmental impacts detected as far downstream as the Kafue Hook Bridge, 700km from the mining area (Bäckström and Jonsson, 1996). Recent geo- and hydro-chemical studies have quantified the impact of the mining industry on Kafue river chemistry. Mwale (1994), the Japan International Cooperation Agency (JICA) (1995), Kasonde (1990), Pettersson and Ingri (1999a, 1999b, 2000) and Pettersson *et al*, (2000) all demonstrate the increased concentrations of dissolved and suspended heavy metals in the Kafue river and the marked accumulation of cobalt, copper, iron and manganese within the river sediment (Table 4). Isakson and Säfvestad (1996), found a marked increase in heavy metal concentrations in sediments corresponding in age to the 1930s, concluding that metal accumulation within the Kafue ecosystem has been occurring since the onset of commercial mining 70 years ago. Aerial photographs from some mining areas clearly show the marked suspended solid loads to the Kafue river and its tributaries, due to unsuitably treated mine effluent entering these waterways through tailings impoundments and pollution control facilities.

Table 4: Dissolved, suspended and sediment cobalt, copper, manganese and sulphur concentrations in the Kafue river upstream, within and downstream of the mining area. Data from Pettersson and Ingri, (1999a, 1999b, 2000) and Pettersson *et al*, (2000).

Metal	**Location relative to the Mining Area**			
	Upstream	**Within**	**Downstream**	**World Average River**
	Dissolved (µmol/l)			
Co	0.003-0.026	0.364-0.429	0.020-0.030	0.003
Cu	0.01-0.04	0.63-2.28	0.30-0.88	0.024
Mn	0.11-0.21	1.57-2.40	0.02-0.09	0.015
S	39-54	2572-760	421-1378	60
	Suspended (ppm)			
Co	66-84	1146-3565	753-1268	20
Cu	316-340	10470-22407	2913-23005	100
Mn	0.5-1.2	0.3-1.8	1.2-6.3	0.1
S	NR	NR	NR	NR
	Sediment (ppm)			
Co	30	1030	595	8
Cu	146	12855	3225	30
Mn	0.07	0.19	0.3	0.1
S	NR	8000	NR	NR

NR: Not Reported

The metal accumulation within the Kafue ecosystem has been associated with various toxicological manifestations such as the disappearance of hippopotami (*Hippopotamus amphibius*) from the Kafue river in Chingola, the proliferation of water hyacinth (*Eichhornia crassipes*) and the bioaccumulation of heavy metals within wildlife liver tissues (Sinkala *et al*, 1997; Syakalima *et al*, 2001). Gilman (1997), in Chingola, and Mwase (1994), in Kitwe, Itezhi-tezhi and Kafue town found elevated levels of copper in river sediment and associated these with increased pathology in fish. Syakalima *et al*, (2001), reports that wildlife (e.g. Kafue lechwe and fish) dependent on the Kafue flats have been exposed to lead levels that have a potential of accumulating thus causing adverse effects. Mwase *et al*, (1998), Norrgren *et al*, (1998) and Norrgren *et al*, (2000) demonstrate increased fish mortality and decreased aquatic productivity following exposure of caged fish, eggs and fry to Kafue river water and sediment from the mining area. Zambia Consolidated Copper Mines (ZCCM), (1982) and Marais, (1981) describe high incidence of livestock mortalities and reproductive problems, and decreased livestock productivity on farms bordering the Mwambashi river (tributary of the Kafue river), and associated the outbreak with copper toxicity.

The role of agrochemicals in the environmental degradation of the Kafue river ecosystem has also been acknowledged. Norrgren *et al*, (2000) report a high concentration of several pesticides, i.e. DDT with its major metabolites, PCBs, and dieldrin in Kafue river water. The study showed that persistent organic pollutants occurred in such high concentrations that they should be considered from ecotoxicological aspects and may affect aquatic animal health. Syakalima *et al*, 2006 demonstrates that there is widespread contamination of the Kafue river with pesticides/herbicides. The pesticides/herbicides detected in water, sediment and fish from the Kafue river collectively included: heptachlor, dichlorodiphenyltrichloroethane (pp'-DDE), cypermethrin, chlordane, toxaphene, terbufos, kelthane, endosulfan, dieldrin, 1-1 dichloro-2, 2 bis (p-chlorophenyl) ethane (pp'-DDD), DDT, atrazine, disulfoton, d-trans-allethrin and endrin. The study also demonstrates that organochlorides are found throughout the river and in fish samples and that the fish expressed oxidative stress as determined by catalase levels. Another study by Sichilongo and Torto, 2006 found that there was a problem of contamination of the Kafue lechwe by suspected endocrine disruptors such as deltamethrin, heptachlor, aldrin, d-t-allethrin, endosulfan, pp-DDE, dieldrin, endrin, pp-DDD and DDT in the Lochinvar National Park.

The study demonstrate that there is a problem of contamination of wildlife by suspected endocrine disruptors in the Lochinvar National Park of Zambia meaning that there is a potential risk to humans living in and on the peripheral of the park. Alsterhag and Petersson (2004), reports that continuous discharge of raw sewerage into Kafue river from KSTP has contributed to the steady supply of nutrients (ortho-phosphates, nitrates, ammonia) ensuring the proliferation of various types of weeds, like Kariba weed (*Salvina molesta*) thereby causing eutrophication.

This study therefore investigated biomarkers of pollution to, polycyclic aromatic hydrocarbons (PAHs), PCBs, 2, 3, 7, 8 tetrachlorodibenzo-p-dioxin (TCDDs), DDT, organochloride pesticides and heavy metals. The biomarkers investigated included; Cytochrome P450 1A1 (CYP 1A1), Cytochrome P450 3A (CYP 3A) and Metallothionein 1 (MT 1) in Kafue lechwe.

1.3 Rationale for the Study

The Kafue river basin of Zambia is one of Africa's most diverse ecological habitats. However, because of its location, a lot of influences from industries, mining and agricultural activities have been documented exposing the basin to potential destruction. Unique to this habitat is the Kafue lechwe, naturally found nowhere else in the world. It is one of the most hunted species for consumption and so has immense ecological, economical and health impact to the nation and local communities. Unfortunately, there has been a decline in the population of this unique species. The Kafue lechwe were about 100,000 up to the time the hydropower station at the Kafue Gorge was being constructed in 1971. Observations that followed after 1975 indicated a decline in the Kafue lechwe population to 80, 000 and 41 000 in 1982 (Kamweneshe *et al*, 2002). In 2001, the population had declined to 40,000 (WWF, 2001). This decline was attributed to a reduction in the area of habitation as a result of the introduction of two physical stressors: 1) hydropower station and 2) Itezhi tezhi dam upstream which are meant to preserve water for power generation in the dry season when the water level is low in the Kafue river. In addition, diseases like tuberculosis (Zieger *et al*, 1998) and brucellosis, poaching, grazing pressures (Kapungwe, 1993), an increase in agricultural activities in the area and chemical

stressors have also been identified as contributors to the overall factors that have led to the population decline. Despite pollutants being well documented in the Kafue river, there has never been a comprehensive risk assessment of the National Parks in the Kafue Basin in order to characterize the impact of these chemical stressors (metals, pesticides and other persistent compunds) on the Kafue lechwe whose water source is the Kafue river that has been reported to have higher than normal levels of pollutants compared to the average world river (Gilman, 1997; Petterson and Ingri, 1993). A lot of information on the effect of all other factors contributing to the decline in the population other than chemical stressors is well documented. This work intends to add to the knowledge gap that exists on the role played by chemical stressors through biomarker studies. From literature it has been observed that biomarkers are powerful tools for studying contaminant exposure and effects on living organisms. Biomarkers are well developed worldwide but they have rarely been used in assessing environmental health in Zambia, especially so among the dependants of the polluted waters of the Kafue river where a lot of work exists on quantitative and qualitative amount of pesticides, other persistent environmental pollutants and heavy metals.

1.4 Objectives

1.4.1 General Objective

The main objective of this study was to assess the potential of using CYP 1A1, CYP 3A and MT 1 as biomarkers of contamination of the Kafue lechwe to pollution in the Kafue river

1.4.2 Specific Objectives

The specific objectives were to:

I. Identify and read the partial sequences of CYP1A1, CYP 3A and MT 1 genes in Kafue lechwe which would lead us to clarifying their involvement in xenobiotic metabolism and activation

II. Define genetic relationships of CYP1A1, CYP 3A and MT 1 of Kafue lechwe and known CYP 1A1s, CYP 3As and MT 1s of other animals

III. Investigate PAH, PCB and TCDD exposure through the use of messenger ribonucleic acid (mRNA) expression level of CYP 1A1

IV. Investigate DDT and other pesticide exposure through the use of mRNA expression level of CYP 3A

V. Investigate heavy metal exposure through the use of mRNA expression level of MT 1

VI. Compare the mRNA expression levels of CYP 1A1, CYP 3A and MT 1 of Kafue lechwe between Blue Lagoon and Lochnivar Game Management Areas

VII. Verify whether expression of CYP 1A1, CYP 3A and MT 1 mRNA in Kafue lechwe can be useful biomarkers of environmental pollutants

CHAPTER 2.0 LITERATURE REVIEW

2.1 Introduction

Pollution of rivers and dams has been largely monitored by periodic sampling of water and comparisons of its physical and chemical characteristics. This approach suffers from the fact that results relate to the time when sampling was done, yet there may be great variability in the pollutant levels due to the discontinuity of pollutant discharge. Moreover, chemical analysis provides little information on the impacts of compounds that are rapidly metabolized and excreted by organisms (Arcand-Hoy and Metcalfe, 1999). There may also be a large range of pollutants within a river depending on the environments through which it passes and in such a case it is not possible to measure all of these and to define their impact on the riverine health. Chemical analyses also are unable to quantify bioavailability of the chemicals to the biological receptor and do not provide any indication of deleterious effects of contaminants on the biota (De Coen, 1999). The chemical methods are also gauged "difficult to perform" and sometimes require extensive sample clean up (United States Environmental Protection Agency (USEPA), 1986) and are also relatively expensive. Assessment also used to rely heavily on mortality-based acute toxicity tests but it has been shown that a chemical that does not cause death within the duration time may still have long term deleterious impacts on the test organisms (Wo *et al*, 1999). The biological approach is now being used to complement chemical methods. Biological methods are based on qualitative and quantitative observations of living organisms in their natural environment (Smolders *et al*, 2003) and the use of biomarkers has recently been proposed to evaluate the effects of pollutants, such as heavy metals and organic xenobiotic compounds in animals (Viarengo *et al*, 1997).

2.2 Assessment of Environmental Pollution in Animals using Biomarkers

Biomarkers are defined by Peakall (1994) as a change in a biological response (ranging from molecular through cellular and physiological responses to behavior changes) that can be related to exposure to/or toxic effects of environmental chemicals. While considering the effect of potential hazards in a biological system, a biomarker is redefined as any biological response measured inside an organism at molecular, biochemical or cellular levels or in its products (urine, feces, feathers and others) indicating a departure from the normal status as a result of exposure to potential toxic chemicals (McCarthy and Shugart, 1990; Van Gestel and Van Brummelen, 1996). Researchers still use different definitions of biomarkers but the one of McCarthy and Shugart (1990) and Van Gestel and Van Brummelen, (1996) seems to be the most commonly used definition. Biomarkers can be used to assess the health status of organisms and to obtain early warning responses of environmental risks (Payne *et al*, 1987). The National Research Council (NRC), 1987 subdivided biomarkers into those of exposure, effect and susceptibility.

A biomarker of exposure refers to an exogenous substance or its metabolite, or product of an interaction between a xenobiotic agent and some target molecule or cell, which is measured in a compartment within an organism. Biomarkers of exposure can be used to confirm and assess the exposure of an individual or population to a particular substance (group), by providing a link between external exposure and internal dosimetry (Van Der Oost *et al*, 2003).

A biomarker of effect refers to a measurable biochemical, physiological or other alterations within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease (Stegeman *et al*, 1992; Van Gestel and Van Brummelen, 1996). Biomarkers of effect can be used to demonstrate either preclinical alterations or adverse health effects due to external exposure and absorption of a chemical.

A biomarker of susceptibility refers to an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance. This includes genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure (Van Der Oost *et al*, 2003). Biomarkers of susceptibility help to

elucidate variations in the degree of response to toxicant exposure observed between different individuals.

Advantages of using biomarkers in pollution monitoring according to Livingstone (1993) include:

a) A temporary and spatial integrated measure of bioavailable pollutants is provided by biomarker

b) Some biomarkers show very specific responses and through this attribute exposure and risk to environmental pollutants

c) By applying different biomarkers to species from different habitats and different trophic habitats, they help establish the importance of different routes of exposure

d) They can provide information on the relative toxicities of specific chemicals and effluents and

e) Biomarkers are applicable in the laboratory as well as in the field

Adams *et al,* (2001) identified three types of biomarkers based on specificity, sensitivity and level of organization. They state that biomarkers range from;

a) General (e.g. stress proteins) to specific (e.g. acetylcholinestrase)

b) Relative low sensitivity (e.g. histopathology) to high sensitivity (e.g. cortisol)

c) Molecular to individual level of biological organization.

A set of biomarkers at levels below that of an individual (molecules, organelles, cells and tissue) will be reviewed here. These are more specific biological and biochemical assays and they include: CYP 1A1, CYP 3A and MT 1.

2.2.1 Cytochrome P450 (CYP 450) as a Biomarker

A lot of biomarkers have been used to evaluate environmental pollution and mostly the phase 1 cytochrome P450 monooxygenase or mixed-function oxygenase (MFO) system have been extensively used. Isoenzymes of CYP 450 are of central importance in the metabolism of many xenobiotics and endogenous compounds and are the commonly studied biomarkers. CYP 450 enzymes are classified as a b-type haemoprotein (haem skeleton of this type also has haemoglobin, myoglobin and certain peroxidases) associated with membranes of the endoplasmic reticulum. This enzymatic system appears ubiquitous and has been found in a vast array of organisms including bacteria, fungi, plants, insects, fish, birds, and mammals (Nelson *et al*, 1996). In eukaryotic cells, it is also bound to mitochondrial membranes and, in bacteria, it is present in the cytosol in a soluble form. In mammals, cytochromes are present, at high levels, in the liver, accounting for 1 to 2% mass of hepatocytes (Lester *et al*, 1993; Lewis, 2001). However, they are also found in the intestine, kidney, lungs, brain, skin, prostate gland, and placenta (Anzenbacherova and Anzenbacher, 1999; Arukwe, 2002; Ortiz-Delgado *et al*, 2002). The biological significance of mammalian CYP 450 enzymes had been recognized already in 1964, when Omura and Sato first described the differential spectrum of dithionite reduced and carbon monoxide complexed cytochromes (Omura and Sato, 1964). In 1996, Nelson and others presented the first systematic approach in allocating individual cytochromes to phylogenetic families and subfamilies (Nelson *et al*, 1996). The CYP 450 superfamily is currently composed of more than 500 genes and pseudogenes belonging to over 200 gene families and these numbers are likely to continue to evolve (Nelson *et al*, 1996). Substrates of the CYP 450 enzymes are many and diverse, including both xenobiotics such as environmental pollutants and drugs, and endogenous compounds such as steroids, fatty acids, and prostaglandins (Nebert and Gonzalez, 1987; Porter and Coon, 1991; Nelson *et al*, 1996; Stegeman and Livingstone, 1998).

Foreign chemicals can undergo two types of biotransformation reactions once present in the body and phase 1 reactions are predominant in the biotransformation process and involve oxidation, reduction, and hydrolysis. They participate in a large number of different oxidation reactions, including hydroxylation, H- and O-dealkylation,

deamination, sulphoxidation, N-oxidation, dehalogenation, e.t.c. (Anzenbacherova and Anzenbacher, 1999). These reactions typically result in the transformation of xenobiotics into metabolites that can undergo phase 2 reactions where compounds are conjugated with an endogenous molecule (glucuronate, glutathione, 3' phosphoadenosine-5'-phosphosulphate, e.t.c.) becoming more hydrophilic and therefore more easily excreted from the body. Metabolites produced in the first phase are eliminated only exceptionally. The CYP 450s are the most important enzyme systems in phase 1 reactions and play a key role in the detoxification of many chemicals. Paradoxically, the CYP 450s can also produce metabolites that are more toxic than the parent compounds (a process known as bioactivation) and such is the case for several PAHs. The electrons required for the catalytic cycle are delivered by the reduced form of Nicotinamide adenine dinucleotide phosphate (NADPH). The catalytic sequence of phase 1 biotransformations via the MFO system follows a reaction cycle, which can be divided into several steps, as illustrated in Figure 4. In the first step, the substrate binds to the prosthetic heme ferric iron (Fe^{3+}) of the enzyme. The complex is then reduced by electron transfer from the flavoprotein NADPH cytochrome P450 reductase to the ferrous state (Fe^{2+}). Subsequently, molecular oxygen is bound to it, a critical point at which catalysis may proceed or be interrupted, resulting in the release of active oxygen (superoxide complex). The next steps involve the addition of a second electron (second reduction), usually via cytochrome b5 (cyt b5), and the formation of a peroxide. This is followed by cleavage of the O-O bond leading to formation of water and the substrate radical complex. The iron-ligated oxygen atom is transferred to the substrate, forming a hydroxylated form of the substrate. Finally, the product is released from the active site of the enzyme, which then returns to its initial state.

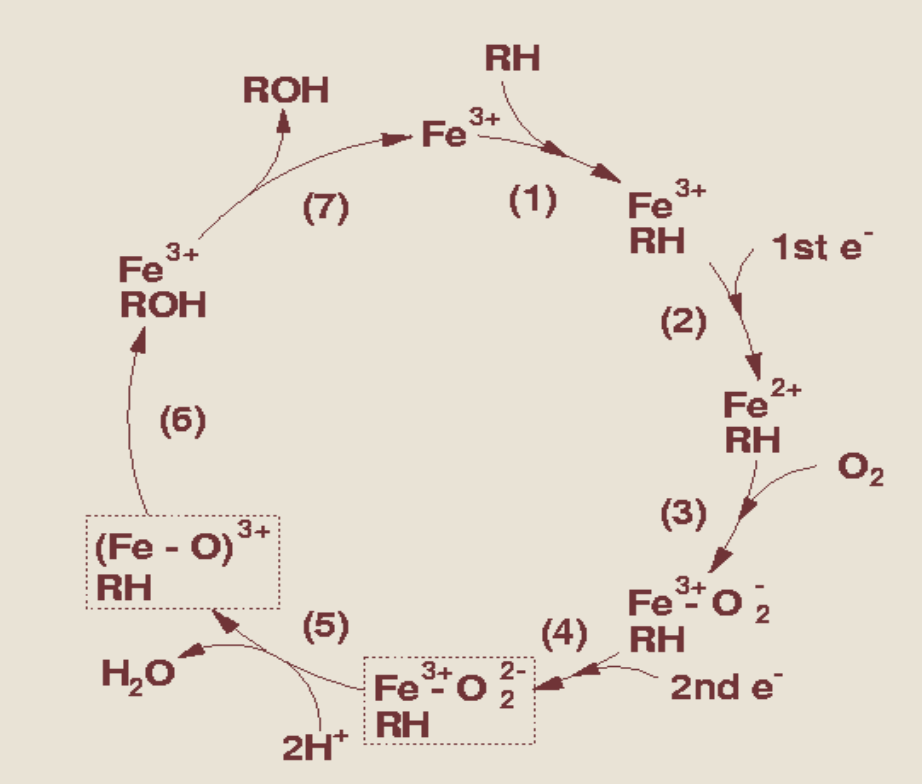

Figure 4: Cycle of CYP 450. Modified from Dawson, (1988) and Werck-Reichhart and Feyereisen, (2000).

In a simplified form, the general catalytic reaction of cytochrome P450 is presented in the following formula:

Substrate (RH) + H^+ + NADPH + O_2 → Product (ROH) + H_2O + NADP

The resulting hydroxylated product may be less toxic than the parent compound, or can be an intermediate of a final product which is more toxic than the parent compound, as seen in the process involved in carcinogenesis (Stegeman and Hahn, 1994; Bartsch *et al*, 2000).

Of the numerous CYP families identified, CYP 1-4 families are induced by xenobiotic chemicals and the induction is mediated by specific or multiple receptors such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptor (PPAR) (Schmidt and Bradfield, 1996; Waxman, 1999; Nebert and Russell, 2002). Therefore, CYP 1-4 families are of particular importance as biomarkers of chemical contamination (Nebert and Russell, 2002).

The CYP 1 family is comprised of CYP 1A, CYP 1B and CYP 1C enzymes and is important in the metabolism and activation of PAHs, PCBs, and aromatic amines and amides. CYP l enzymes can also be induced by chlorinated dioxins and furans. The CYP 2 family consists of 15 gene subfamilies. CYP 2 proteins involved in xenobiotic metabolism mostly include CYP 2B, CYP 2C, CYP 2D, and CYP 2E enzymes. CYP 2Bs metabolize barbiturates, PCBs, chlorinated pesticides, and many tumor promoters. CYP 2C9 and CYP 2Ds are important in the metabolism of pharmaceutical drugs such as ibuprofen, tolbutamide, S-warfarin, codeine, debrisoquine, and oxyprenolol (Smith and Jones, 1992). CYP 2E enzymes metabolize small molecules such as ethanol, acetone, benzene, and alkylnitrosamines. CYP 3As metabolize large and highly lipophilic xenobiotics such as macrolide antibiotics (erythromycin), cyclosporine, DDT and some PAHs (Maurel, 1996) while CYP 4 enzymes are involved in the metabolism of carboxylic acids (arachidonic acid) and are induced by antilipidaemic drugs such as clofibrate (Parke *et al*, 1991).

In the CYP 1A subfamily, genes of two members, CYP 1A1 and CYP 1A2 have been identified and CYP lAl has been studied in a large number of species and in various taxa (invertebrates, amphibians, fish, birds, mammals) and its induction is widely used as a biomarker of exposure to PAHs, PCBs and polychlorinated dibenzo-p-dioxin (PCDD) (Goksøyr, 1995). In cetaceans, levels of CYP 1A1 expression in liver and blubber of beluga whale (*Delphinapterus leucas*) were correlated to concentrations of mono-ortho and non-ortho PCBs (known inducers of CYP 1A1) (White *et al*, 1994). CYP 1A activity has been found to be relatively high in the tissues of polar bears in the Canadian Arctic (Muir *et al*, 1999), and the authors suspect that non-ortho and mono-ortho PCBs were responsible for this activity. In rodents, CYP 1A1 expression has been correlated to higher order toxic effects including thymic atrophy, weight loss and toxicity induced by PCB, PCDD and polychlorinated dibenzofuran (PCDF) exposure (Poland and Knutson, 1982; Safe, 1987; Safe, 1990). Previous studies on the fish *Chionodraco hamatus* and *Trematomus bernacchii* exposed to PAHs demonstrated a significant induction of CYP 1A1 (Focardi *et al*, 1995; Regoli *et al*, 2005). Therefore, the induction of CYP 1A has been used to indicate the exposure of organisms to inducing agents, as well as to evaluate the degree and possible risk of environmental contamination (Stegeman *et al*, 1992). As such, the importance of CYP 1A induction is to enhance the biotransformation of the compounds. In the absence of an inducer, the transcription of the CYP 1A gene is repressed by a

repressor protein, which accounts for the low constitutive levels of CYP 1A in most of the species. The induction mechanism of CYP 1A is illustrated in Figure 5 and involves both depression and activation of transcription by the aryl hydrocarbon receptor (AhR). The AhR is normally complexed in a 1:2 ratio with heat shock protein kD 90 (hsp90), which dissociates upon binding of ligand to the AhR, enabling the receptor to be phosphorylated by the tyrosine kinase. The activated AhR then enters the nucleus and forms a heterodimer complex with the AhR-nuclear translocator (Arnt). Inside the nucleus, the AhR-Arnt complex binds to regulatory sequences known as dioxin-responsive elements (DRE) or xenobiotic responsive elements (XRE) and enhances the transcription of the CYP 1A gene and other genes with an XRE or XRE-like sequence in their upstream enhancer region. The CYP 1A mRNA is translated to protein in the ribosomes. The heme binds to the protein and the enzymatic function occurs when the enzyme is inserted into the membrane of the endoplasmic reticulum. In mammals, the battery of AhR-responsive genes include CYP 1A1, CYP 1A2, CYP 1B1 and some phase 2 enzymes such as uridine 5-diphosphate-glucuronyl transferase (UDP-GT) and glutathione- S-transferases (GST) (Nebert *et al*, 2000).

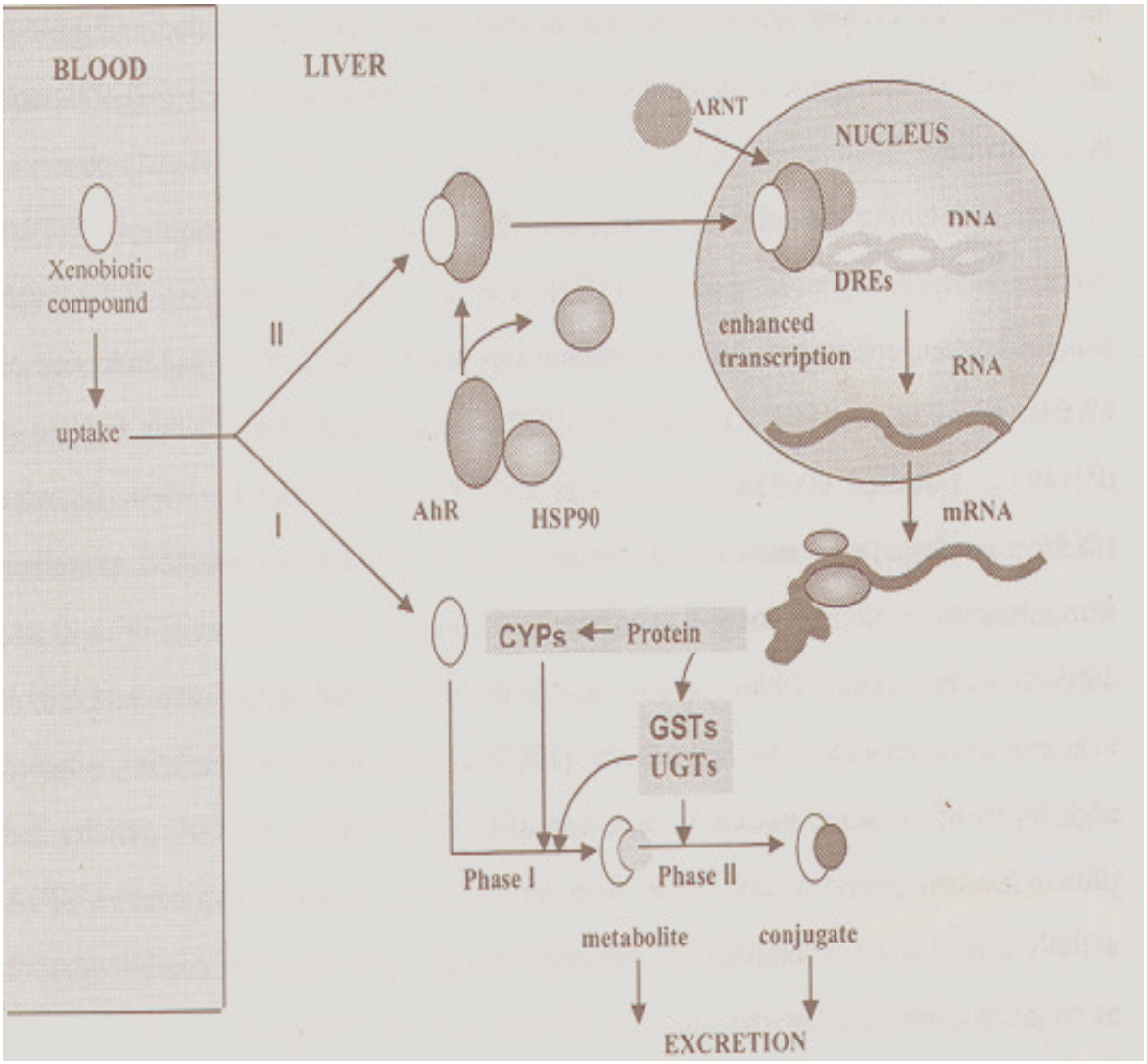

Figure 5: Simplified presentation of the fate of xenobiotic compounds in the liver cell. Route I, a possible mechanism for detoxification or intoxication and route II, a possible mechanism for enzyme induction (Stegeman and Hahn, 1994).

The CYP 3 gene family is believed to have diverged between 800 and 1100 million years ago (Nebert and Gonzalez, 1987; Gonzalez, 1990). This gene family has two identified subfamilies, CYP 3A and CYP 3B, the latter recently discovered in the pufferfish (*Takifugu rubripes*) genome. The CYP 3A subfamily is one of the largest in the CYP 450 superfamily and multiple CYP 3A genes have been identified in several species (Nelson *et*

al, 1996). It has been most thoroughly investigated in mammals (Maurel, 1996) and has multiple members in several vertebrate species, which in human and rat is clustered on chromosomes 7 and 6, respectively (Simmons *et al*, 1985; Brooks *et al*, 1988; Spurr *et al*, 1989). The functional diversity of the CYP 3A subfamily is extraordinary. Over 50% of all drugs in use today are substrates and/or inhibitors of mammalian CYP 3A enzymes. In addition to therapeutic drugs, CYP 3A enzymes also are involved in the metabolism of endogenous hormones, bile acids, fungal and plant products (Maurel, 1996; Thummel and Wilkinson, 1998; Guengerich, 1999) and environmental pollutants such as DDTs and its metabolites (Nims and Lubet, 1995). CYP 3A activities thus provide a broad biochemical defense against bioaccumulation of endogenous and exogenous lipophilic compounds to toxic levels. CYP 3A enzymes also can activate several pro-carcinogens and are therefore believed to be involved in chemical carcinogenesis (Guengerich and Shimada, 1991). CYP 3A is also an inducible enzyme found predominantly in liver and in gastrointestinal tract of mammal and teleost species (Lee *et al*, 2001). In tilapia (*Oreochromis niloticus*), levels of CYP 3A expression in liver have been attributed to exposure to chemicals such as PCBs, organochlorine pesticides and PAHs in the billings reservoir (Bainy *et al*, 1999). Recently, Pathiratne and George (1996) observed a 2-fold induction of CYP 3A in tilapia treated with organic compounds, polychlorinated naphthalenes (PCN). Previous studies on common minke whales (*Balaenoptera acutorostrata*) exposed to organochlorine contaminants (OCs) demonstrated a significant induction of CYP 3A (Niimi *et al*, 2007). Another study demonstrated that zebrafish CYP 3A65 transcription was enhanced by 2, 3, 7, 8- TCDD (Tseng *et al*, 2005). Recently, the molecular mechanisms by which structurally diverse xenobiotics activate CYP 3A genes have been resolved. The xenobiotics-inducible CYP 3A expression is modulated by the PXR (Smirlis *et al*, 2001; Xie *et al*, 2000; Wei *et al*, 2002) (Figure 6). The PXR also called steroid and xenobiotic receptor (SXR) is a recently identified orphan nuclear receptor (Kliewer *et al*, 1998). The non-liganded receptor are localized in cytoplasm with cytoplasmic heat shock protein 90 complex, and translocate into the nucleus following the treatment with phenobarbital type (PB-type) xenochemicals (Kawamoto *et al*, 1999; Kawana *et al*, 2003; Kobayashi *et al*, 2003; Squires *et al*, 2004). The ligand-activated receptor forms a heterodimer with retinoid X receptor (RXR) and binds to specific response elements xenobiotic responsive enhancer module (XREM) in CYP 3A gene (Honkakoski *et al*, 1998; Goodwin *et al*, 2002; Sueyoshi

et al, 1999; Wang *et al*, 2003; Faucette *et al*, 2006). Further recruitment of coactivators including steroid receptor coactivator 1 to PXR/ RXR complex results in the induction of CYP 3A genes (Forman *et al*, 1998; Min *et al*, 2002). Despite the central role of the CYP 3A enzymes in drug metabolism, the reactions they catalyze have rarely been used as biomarkers.

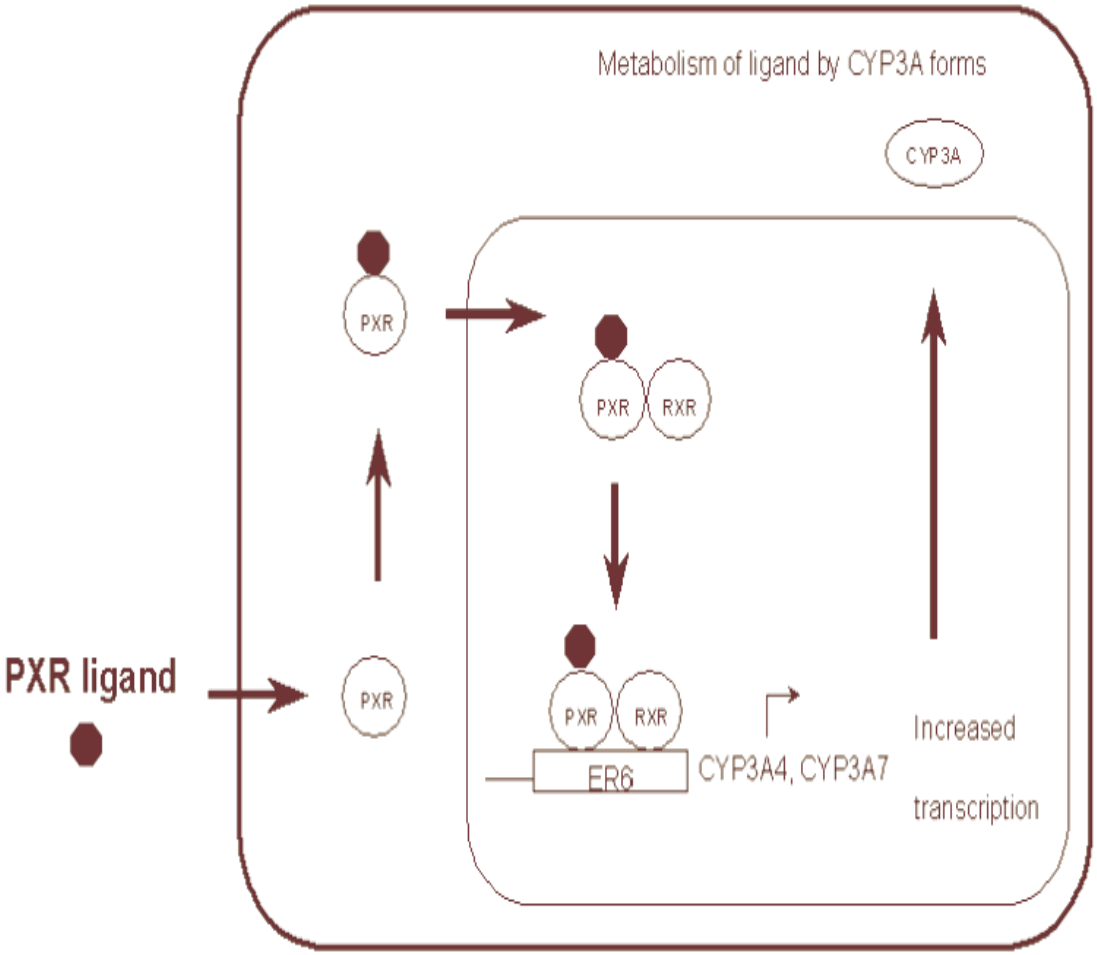

Figure 6: Schematic model of a xenobiotic binding and activating PXR leading to CYP 3A4 induction (Tompkins and Wallace, 2007).

The toxicological examination of CYP 450 includes measurement of the catalytic activity of the cytochrome arising in response to xenobiotic exposure. The CYP 1A preferentially catalyses the induction of O-dealkylation of 7-methoxyresorufin and 7-ethoxyresorufin. Thus a common method of examining the responses of CYP 1A isoenzyme is to determine its catalytic activity through ethoxyresorufin O-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH) activities. Whereas the EROD assay measures the increase in fluorescence of the reaction product resorufin (Burke and Mayer, 1974), the AHH measures the hydroxylation of PAHs, such as benzo[a]pyrene (BaP). The fluorescent product formed can be detected by several methods, of which fluorescence spectrofluorometry is the most common. In addition to measuring the catalytic activities, the CYP 1A proteins can be measured by immunological assays using mono and polyclonal antibodies with enzyme-linked immunosorbent assay (ELISA), western blot and immunohistochemistry techniques (Bucheli and Fent, 1995). Since the elevation of CYP 1A proteins is preceded by synthesis of mRNA, determination of mRNA is also a useful approach of determining exposure of an individual to CYP 1A inducing agents. It may be measured using northern blot and CYP 1A complementary deoxyribonucleic acid (cDNA) probes by reverse transcription polymerase chain reaction (RT-PCR) techniques (Bucheli and Fent, 1995; Rabergh *et al*, 2000; Rees and Li, 2004). Techniques measuring the induction of CYP 1A through quantification of CYP 1A protein and mRNA levels are particularly useful for detecting CYP 1A induction caused by chemicals that bind tightly to the active site (Parkinson, 2001). In addition, the CYP 1A inducing potential of environmental pollutants can also be analyzed by in vitro assays using transgenic cell line (Anderson *et al*, 1996). The CYP 3A on the other hand, preferentially catalyses the induction of testosterone 6β- hydroxylation detected by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). Other examinations that have been described for CYP 1A1 can also be applied in the determination of CYP 3A.

2.2.2 Metallothioneins (MTs) as a Biomarker

Metallothioneins (MTs), the family of metal binding proteins are one of the most promising biochemical indicators for exposure to heavy metals (Hamilton and Mehrle, 1986; Haux and Forlin, 1988). This class of proteins was discovered in 1957 by Margoshes and Valee as a part of an extract from the horse kidney (Margoshes and Vallee, 1957). Currently there are four groups of metallothioneins (MT 1, MT 2, MT 3 and MT 4) known according to the Expert Protein Analysis System (ExPASy) Proteomics Server and MT 1 and MT 2 are coordinately regulated by metals (Palmiter, 1987). Biochemical characteristics of MTs include that: (1) they are of low molecular weight 6 to 10 kD (Petrlova *et al*, 2006; Studnickova *et al*, 1997) and non-enzymatic proteins; (2) they are soluble, sulphnydryl-rich proteins; and (3) they have peculiar cysteinyl-rich amino acid sequences such as Cys-X-Cys, Cys-XY-Cys where XY are amino acids different from cysteine (Kagi and Kogima, 1987). The Cysteinyl residues make up 25-30% of the protein and serve as ligands for metal chelation. They have a high heavy affinity and binding capacity (7 to 9 g atom per mole thionein) and are able to chelate both essential metals such as Zn and Cu and non-essential metals such as cadmium (Cd), mercury (Hg) and silver (Ag) by cysteine tetrathiolate clusters (Viarengo *et al*, 1999). MTs show different affinities for heavy metals (Hg>Ag>Cu>Cd>Zn).

Their physiological functions are still unclear (Klaassen *et al*, 1999) but they are thought to function in the transport, storage and detoxification of heavy metals (Klaverkamp *et al*, 1991; Kagi and Shaffer, 1988). Several functions of MTs have been proposed. They are thought to play an important role in essential metal homeostasis. For example, MTs might be essential in Zn homeostasis by regulating Zn absorption or as a donor of Zn to various enzymes and transcription factors during development or protein synthesis (Klaassen *et al*, 1999). They might also protect against Cu toxicity by sequestration of Cu from critical cellular targets. They also play a role in Cd disposal and detoxification. Cd is toxic to a number of tissues and acute exposure produces hepatic, pulmonary and testicular injury whereas chronic exposure results in renal and bone injury and cancer. Another function of MTs is to protect against Hg toxicity in the cytosol (Liu *et al*, 1991). They also serve as sacrificial scavengers for reactive oxygen species (ROS e.g. hydroxyl radicals and superoxide anion) (Maret, 1994; Maret and Vallee, 1998). Other toxic stresses that MTs

protect against are tert-butylhydroperoxide and hydrogen peroxide (Abel and Ruiter, 1989) and irradiation (X- and Ultraviolet rays). It is proposed that MTs in the cytosol might function in metal detoxification and protection from oxidative stress while those in the nucleus may provide protection against DNA damaging electrophiles (Woo and Lazo, 1997). MTs can be found mostly in liver, kidney, pancreas and intestines of animal species.

MTs are inducible proteins and heavy metal cations accumulated within cells stimulate MT neosynthesis (Figure 7) by enhancing MT gene transcription (Andersen and Werser, 1979). The promoter regions of metallothionein genes contain the so-called metal-responsive elements (MRE's) which are responsible for induction by heavy metals (Stuart *et al*, 1985; Serfling *et al*, 1985). In 1988 the factor binding to the MRE promoter sequences was described as MTF-1 (metal-responsive element- binding transcription factor-1 or, for short, metal transcription factor-1). It required elevated zinc concentrations for optimal DNA binding (Westin and Schaffner, 1988). MTF-1 is a ubiquitously expressed zinc finger protein that is essential for basal and heavy metal-induced expression of metallothioneins (Heuchel *et al*, 1994). Therefore, MTF-1 is the key regulator of metallothionein expression (Günes *et al*, 1998; Simpkins, 2000). MT 1 and MT 2 are well studied MTF-1 genes. When cells are treated with heavy metals, MTF-1 is activated, binds to MRE's and induces transcription of target genes, notably metallothioneins (Durnam and Palmiter, 1981; Radtke *et al*, 1993; Auf der Maur *et al*, 2000). In resting cells, most MTF-1 localizes to the cytoplasm whence it is translocated from the cytoplasm to the nucleus under several different stress situations like heavy metal load (Smirnova *et al*, 2000; Saydam *et al*, 2001). The finding that MTF-1 requires an elevated concentration of zinc for strong binding to deoxyribonucleic acid (DNA) suggests that MTF-1 is activated by allosteric regulation of DNA binding via binding of metals to the transcription factor itself (Radtke *et al*, 1993; Heuchel *et al*, 1994; Westin and Schaffner, 1988). Although other heavy metals readily induce metallothionein gene transcription in cultured cells, none of them can substitute for Zn in a cell-free DNA binding reaction of MTF- 1 (Heuchel *et al*, 1994). The most likely scenario is replacement of Zn by these other heavy metals in cellular and/or extracellular Zn-storage proteins, leading to concomitant activation of MTF-1 by the released Zn. In addition, MTF-1 can be phosphorylated upon metal induction, as a result of the activation of a complex kinase signaling transduction pathway which includes protein

kinase C (PKC), phosphoinositol- 3 kinase (PI3K), c-Jun N-terminal kinase (JNK) and a tyrosine-specific kinase (LaRochelle *et al*, 2001). This suggests that metal ions such as Cd could also activate MTF-1 by stimulating kinases.

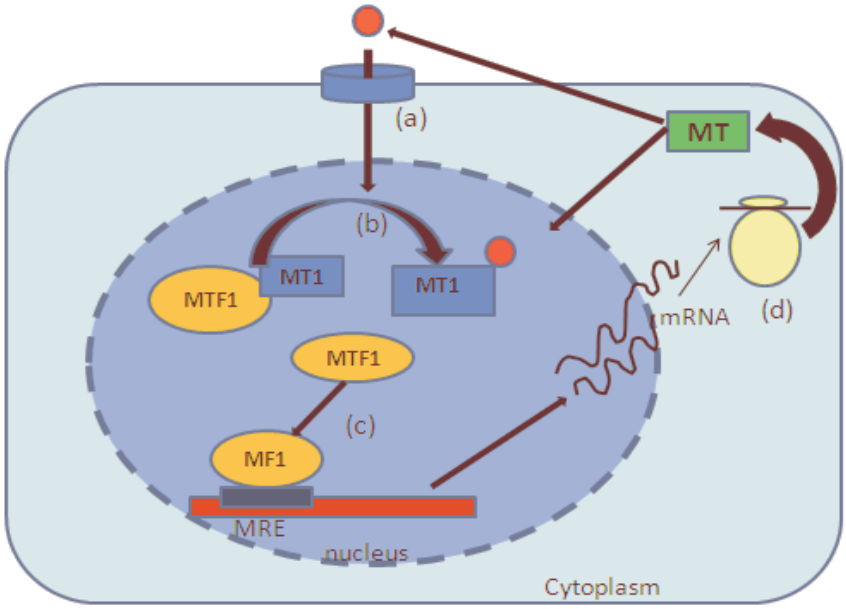

Figure 7: Schematic model of a heavy metal binding and activating MTF-1 leading to MT induction (Beklova *et al*, 2008)

MTs are usually considered an important specific biomarker of heavy metal exposure due to their inducibility by heavy metals (Viarengo *et al*, 1999). The importance of MTs in biomonitoring is enhanced by their ubiquity which means they can be studied in most organisms (Hamer, 1986). These proteins are abundant through the whole animal kingdom, and they have also been found in higher plants, eukaryotic microorganisms and some prokaryotes. In several teleost species, MT levels have been demonstrated to increase in dose dependent manner after administration of heavy metals (Hogstrand and Haux, 1991; George and Young, 1986; Sandvik *et al*, 1997). Several field studies with feral fish also supported the experimental data that MT levels correlate with levels of heavy metals measured in tissues (Hylland *et al*, 1992; Campana *et al*, 2003; Chowdhury *et al*, 2004). MTs have been examined in a natural population of oysters in the Patuxent river, Maryland, a metal-contaminated tributary of the Chesapeake Bay (Wright and Zamuda, 1991). MTs have been demonstrated in marine crustaceans exposed to elevated levels of cadmium and copper in their water and food (Olafson *et al,* 1979a, 1979b; Overnell and

Trewhelia, 1979; Rainbow and Scott, 1979; Overnell, 1982; Wiedow *et al*, 1982; Engel and Brouwer, 1984b; Engel *et al*, 1985; Engel and Brouwer, 1986; Overnell, 1986). In the blue crab (*Callinectes sapidus*), MTs have been shown to bind cadmium, copper, and zinc in both laboratory exposed animals (Brouwer *et al*, 1984; Engel *et al*, 1985) and in animals from contaminated environments (Weidow *et al*, 1982; Engel and Brouwer, 1984b).

Different methods have been developed for MT evaluation that include: chromatographic separation of soluble cytosolic MT- containing fraction associated with the evaluation of metal concentration (Suzuki, 1980; Lehman and Klaassen, 1986), metal substitution assays (Martinez *et al*, 1993; Loebel and Payne, 1987,) and radio immunological techniques (Nolan and Shaikh, 1986; Hogstrand and Haux, 1990). A spectrophotometric method has also been developed and involves the evaluation of the MT concentration in a partially purified metalloprotein–containing fraction obtained by acidic ethanol/chloroform fractionation of tissue homogenate. MT proteins can also be measured by immunological assays using mono and polyclonal antibodies with ELISA, western blot and immunohistochemistry techniques. MT induction can be measured as the concentration or rates of formation of the responsible mRNA and therefore determination of mRNA is also a useful approach of determining exposure of an individual to MT inducing agents. It may be measured using northern blot and MT cDNA probes by RT-PCR.

The measurement of biomarker responses demonstrate that toxicants have entered an organism, been distributed within the tissue, and are eliciting a toxicological effect on biological structures and functions (McCarthy and Shugart, 1990). Ecotoxicologists have realized that biomarkers measured at molecular or cellular level are of great value in environmental quality assessments. Biomarkers applied both in laboratory and field studies can provide an important linkage between laboratory toxicity and field based assessments. Using field samples, biomarker data may provide an important index of the total external load that is biologically available in the exposure environment (Van der Oost *et al*, 2003). Therefore biomarkers are now currently of interest in ecotoxicological research as they provide functional measures of receptor species and exposure to environmental stressors that can be better related to the adverse effects of human activities. This effects-based information can be used to support environmental management and regulation (Adams *et al*, 2001).

CHAPTER 3.0 MATERIAL AND METHODS

3.1 Study Area

Samples for this study were collected from the Kafue lechwe in Lochnivar and Blue Lagoon Game Management Areas (GMAs) starting from June to December 2008. The GMAs are located within the Kafue basin which is a flood plain of about 6,000 km^2 (Ghirotti *et al*, 1991; Munag'andu *et al*, 2006; Siamudaala *et al*, 2003) comprising Lochnivar National Park in the south (410 km^2), Blue Lagoon National Park in the north (420 km^2) and Kafue Basin Game Management Areas (5,175 km^2) (Sheppe,1985), Figure 3.

3.2 Study Design

Convenient sampling was employed in this study. This mode of sampling is ideal because wild animals are provided through hunting quotas and as such only hunter harvested samples can be obtained.

3.3 Sampling Procedure

3.3.1 Sample Size

The Zambia Wildlife Authority (ZAWA) allowed seventy (70) adult Kafue lechwe antelopes of both sexes to be sacrificed for disease surveillance under a special license request. Forty (40) animals were harvested from Lochnivar GMA and thirty (30) from Blue Lagoon GMA. Therefore, sample size estimates were not calculated as we relied on the actual numbers that had been permitted for research purposes by ZAWA. There were no controls for this study as the Kafue lechwe are indigenous to Kafue flats and thus all animals are exposed to similar conditions. However, Kafue lechwe that are located in uncontaminated systems such as game ranches could have been used as controls to determine whether the levels recorded in this study are background levels or responses to contamination.

3.3.2 Sample Collection

Samples were collected from "hunter harvested" lechwe antelopes. Postmortem of the carcasses was conducted as described by Gracey, (1997). Briefly, the postmortem examination consisted of visual inspection and digital palpation of the integument, liver, lungs, diaphragm, and mesentery e.t.c. All the animals were eviscerated and the abomasum, rumen, etc with the intestines processed separately. Greater detail was paid to the liver for its prominent role in xenobiotic and endogenous metabolism. Visual inspection, digital palpation was done before the pieces of liver could be collected. Then the liver samples were collected using forceps and scissors and placed in labeled polythene bags, then wrapped in labeled aluminium foil and preserved in liquid nitrogen. The samples were transported in this state to The University of Zambia laboratory and transferred to a -80°C fridge where they were stored until analysis. Results of gross visual inspection and postmortem examination (Appendix 1.0) of some of the lechwe showed of both endo and ectoparasites and also tuberculosis granulomas in the lungs.

3.4 Laboratory Analysis

From the 70 samples collected only thirty eight (38) samples (twenty three (23) from Lochnivar GMA and fifteen (15) from Blue Lagoon GMA) were analyzed for biomarkers (CYP 1A1, CYP 3A and MT 1) of pollution as follows:

3.4.1 Total RNA Isolation

Total RNA was prepared from each liver sample by the single-step method (Chomczynski and Sacchi, 1987) using total RNA isolation (TRI) reagent from Sigma Chemical Co. (St. Louis, MO, USA).

3.4.1.1 Homogenization

Liver samples which had been stored at -80°C were thawed on ice and 50 to 100mg from each sample was obtained and transferred into eppendorf tubes containing 1mL of (TRI) reagent. The homogenization of the liver tissue in TRI reagent was performed using a hand held tissue grinder until the tissue was completely dissolved in the solution. Once homogenized, the solution was incubated at room temp for five (5) minutes.

3.4.1.2 Phase Separation

After incubation, 0.2ml of chloroform (Wako Pure Chemicals, Tokyo, Japan) was added to the solution and vortexed for fifteen (15) seconds and left at room temperature for ten (10) minutes. The samples were then centrifuged at 15,000xg for twenty (20) minutes at 4°C.

3.4.1.3 RNA Precipitation

Following centrifugation, there were three phases visible within the tubes, the aqueous phase on top of the tube, the protein phase in the middle of the tube and the organic phase at the bottom of the tube. The aqueous phase (top) was collected by pipetting and transferred to fresh eppendorf tubes being careful not to contaminate the solution with the other phases. Contamination would have been obvious by the presence of any flakes or unclear liquid. Then 0.5ml isopropanol (Sigma-Aldrich Co, St Louis, USA) was added to the fresh tube and mixed gently and incubated at room temp for 10 minutes. The samples were then centrifuged at 15,000xg for 10 minutes at 4°C.

3.4.1.4 RNA Wash and Resuspension

Following centrifugation, the supernatant was removed by pipetting and discarded and the RNA pellet obtained at the bottom of the tubes was washed with 1ml of 70 % ethanol (Kanto Chemical Co. INC, Japan) and vortexed. The samples were then centrifuged at 15,000xg for 5 minutes at 4°C. The supernatant was removed and discarded and the remaining ethanol was allowed to air dry for 2-3 minutes. The tubes were transferred to a 55° C heat block and left to sit for 2-3 minutes. The pellet was then redissolved in 50ul of

diethylpyrocarbonate (DEPC) water (Sigma-Aldrich Co, St Louis, USA) to obtain total RNA solution.

3.4.1.5 Quantification and Quality Control

RNA concentration was determined spectrophotometrically (Nanodrop ND-1000) at 260 and 280 nm and the 260/280 ratio was used to check the purity of the RNA (Youdim *et al*, 2007). Total RNA was then stored at -80°C until use.

3.4.2 cDNA Synthesis (Reverse Transcription) and Polymerase Chain Reaction (PCR) Amplification

The cDNA were synthesized from total RNA by reverse transcription (RT). The template RNA solution were removed from the -80°C freezer, and the enzyme reverse transcriptase (ReverTra Ace, Toyobo, Japan) was removed from the -20°C freezer and were thawed on ice. The primer solution (oligo DT Toyobo, Japan), 5-fold concentrated reverse transcriptase (RT) buffer (Toyobo, Japan), 2.5 mM deoxyribonucleotide triphosphate (dNTP) mixture (Toyobo, Japan), and DEPC water were thawed at room temperature. The individual reagents were then mixed thoroughly to assure homogeneity and then stored on ice immediately after thawing. The reaction mixture was prepared on ice to a final volume of 20µl in each microfuge tube. Each reaction mixture contained the following: 1µg of total RNA and DEPC water which were combined to a final volume of 6.5µl in the tubes. Then 0.5 µl of 10 pmol/ µl oligo DT was added to the tubes, vortexed and then the tubes were spun down and placed in the thermal cycler (Bio-Rad icycler^{TM} Thermal Cycler). The mixture was denatured at 70°C for 10 minutes and then the tubes were placed on ice for 5 minutes. Four micro liters (4µl) of the 5-fold concentrated RT buffer, 8 µl of 2.5 mM dNTP, and 1 µl of reverse transcriptase were then added to the mixture. The tubes were gently tapped and spun down and then placed in the thermal cycler which was programmed as follows: 42°C for 50 minutes and 99°C for 5 minutes. After removal of the tubes from the thermal cycler, DEPC water was added to make 25- fold cDNA. The 25-fold cDNA was then stored at -80°C until use.

The first PCR was performed on Bio-Rad icycler™ Thermal Cycler. The 25-fold cDNA was removed from -80°C freezer and enzyme (Ex taq, Takara Bio. INC, Japan) was also removed from -20°C freezer. They were then thawed on ice. The forward and reverse primers (SIGMA GENOSYS, Japan), 10-fold Ex taq buffer (Takara Bio. INC, Japan), dNTP mixture (Takara Bio. INC, Japan), and DEPC water were removed from -20°C and thawed at room temperature. The individual reagents were mixed to assure homogeneity and were then stored on ice immediately after thawing. The PCR reaction mixture was prepared on ice. The following were pipetted into microfuge tubes to make a final volume of 20 µl: 1.0µl of 10 µM forward primer, 1.0µl of 10 µM reverse primer, 13.3 µl of DEPC water, 1.6µl of 2.5mM dNTP mixture, 2.0 µl of 10- fold Ex taq buffer, 0.1 µl of Ex taq and 1.0µl of 25-fold cDNA. The tubes were then vortexed and then spun down and placed in thermal cycler. The PCR conditions were as follows: denaturation (1 cycle at 94°C for 1 minute), amplification [40 cycles at 94°C for 30sec, gradient annealing temperature of 57°C to 64°C (CYP 1A1 and CYP 3A) and 54.6°C to 61°C (MT 1) for 30 sec and 72°C for 1 min], and final extension (72°C for 5 min). The gradient annealing temperatures were used to determine the optimal annealing temperature. Three microliters (3µl) of each PCR product was then mixed with 1µl loading buffer (Sigma Aldrich Tokyo, Japan) and pipette into wells of 1.5% Agarose gel stained with ethidium bromide (0.25 µg/ml) (Appendix 2.0) in 0.5XTris Boric Acid EDTA (TBE) buffer (Appendix 3.0). Four micro liters (4µl) of 100bp ladder (Sigma Aldrich Tokyo, Japan) was used to run electrophoresis which was performed on a Mupid®-2X submarine electrophoresis system (ADVANCE, Japan).

For the primer design of CYP 1A1, a search of CYP 1A1 sequence for horse 1A1 (gene accession number XM_001493909), pig 1A1 (gene accession number NM_214412), and sheep 1A1 (gene accession number NM_001129905) from National Center for Biotechnology Information (NCBI) was done. Then Clustal W was used to determine the common (conserved) sequence of the three species. The conserved sequence was used as primer because we thought that lechwe also had conserved CYP 1A1 sequence. Therefore, the following primers were used for cloning and to amplify 252bp fragments of CYP 1A1:

- CYP1A-F>5'-CTTTGTGAACCAGTGGCAGA-3'
- CYP1A-R>5'-GGCCAGGAAGAGAAAGACCT-3'

The primers used for cloning and to amplify 500bp of CYP 3A cDNAs were CYP 3A mammalian degenerate primers. The sense primer (5'-TTTGGRGCCTACAGCATGGA-3' where R is for A or G) and the antisense primer (5'-ACYACCATGTCRAGATACTCC-3' where R is for A or G, and Y is for T or C) were both positioned upstream from the well described cysteine-containing heme-binding region (Kawajiri *et al*, 1984; Nebert and Gonzalez, 1987; Gonzalez, 1988; Gonzalez *et al*, 1985). This region has been regarded as unconverted region of CYP 3A in mammals and suggested to be appropriate region for the analyses of CYP 3A sequence.

For MT 1 primers, we used the primer set from a research paper by Wu *et al*, 2007. According to the authors, the primers were designed based on the coding region sequences of MT 1 and MT 2 in the cow (*Bos taurus*), sheep (*Ovis aries*), mouse (*Mus musculus*), rabbit (*Oryctolagus cuniculus*), and pig (*Sus scrofa*) published at the NCBI (http://www.ncbi.nlm.nih.gov/; last accessed Dec. 6, 2006). Therefore, the forward and reverse primers used in this study for cloning and amplification of 183bp fragments MT 1 were:

- Y-MT-SP1>5'-ATGGACCCGAACTSCTCCTGC-3' S=C,G
- Y-MT-SP2>5'-GGCRCAGCAGCTGCACTTGTCCG-3' R=A,G

3.4.3 Direct Sequencing of PCR Products

The sequencing reaction was performed using the PCR products and Big Dye Terminator kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. The enzyme [ready reaction premix including Taq (Applied Biosystems, Japan)] was removed from -20°C freezer and thawed on ice. The forward and reverse primers (SIGMA GENOSYS, Japan), 5 -fold Big Dye Buffer (Applied Biosystems, Japan), and DEPC water were also removed from -20 freezer and thawed at room temperature. The individual reagents were mixed to assure homogeneity immediately after thawing and together with the PCR products, they were then stored on ice. The sequencing reaction mixture was

prepared on ice and contained the following to a final volume of 10 µl in each tube; 2µl of 1µM forward primer or 2µl of 1µM reverse primer, 5.45 µl of DEPC water, 1.75 µl of 5 - fold Big Dye Buffer, 0.5 µl of ready reaction premix including Taq and 0.3 µl of the PCR products (diluted to 60ng/µl with DEPC water) that showed distinct bands on agarose gel electrophoresis. The tubes were vortexed and then spun down and placed in the thermal cycler. The sequencing reaction was performed at 96°C for 15 seconds, and 40 cycles of 96°C for 20 seconds, 55°C for 15 seconds, and 60°C for 4 minutes. Amplification was performed on Bio-Rad icycler™ Thermal Cycler. Ethanol precipitation was performed after the amplification.

3.4.4 Ethanol Precipitation

The products of sequencing reaction were transferred into eppendorf tubes and 64µl of 95% ethanol, 16µl of DEPC was added. The contents were mixed by inverting the tubes and incubated at room temperature for 10 minutes, then centrifuged at 15,000xg for 15 minutes at room temperature. The supernatant was carefully discarded being careful not to disturb the pellet and then 125 µl of 70% ethanol was added and mixed by inverting the tube. Then centrifugation was done at 15,000xg for 10 minutes at room temperature. The supernatant was then carefully discarded being careful not to disturb the pellet. The pellet was then air dried and then dissolved in 12.5 µl formamide (Applied Biosystems, Japan). The contents were then heated at 95°C for 2 minutes using the Iwaki Asahi Techno Glass Thermo Alumi Bath ALB-121 and then cooled on ice. The contents were then transferred into the vials for nucleotide sequence analysis. The nucleotide sequence was analyzed by an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems, Japan) following the manufacturer's instructions. Sequence identities were then confirmed by basic local alignment search tool (BLAST) search against the NCBI sequence database.

3.4.5 Phylogenetic Relationships

The nucleotide sequence of 5 mammalian CYP 1A1s [(sheep (*Ovis aries*), cattle (*Bos taurus*), pig (*Sus scrofa*), horse (*Equus caballus*) and CYP 3A1 of rat (*Rattus norvegicus*) used as an out group)] and 4 mammalian CYP 3A[(sheep (*Ovis aries*), cattle (*Bos*

taurus), pig (*Sus scrofa*), horse (*Equus caballus*), and 1 fish, Killifish (*Fundulus heteroclitus* used as an out group)] and 7 mammalian MT [(sheep (*Ovis aries*), cattle (*Bos taurus*), domestic pig (*Sus scrofa*), pig (*Sus scrofa*), horse (*Equus caballus*), yak (*Bos grunniens*), man (*Homo sapiens*) and whale (*Balaena mysticetus* used as an out group)] were retrieved from the GenBank database (Table 5). The deduced partial nucleotide sequences of the CYP 1A1, CYP 3A and MT 1of Kafue lechwe were aligned with these vertebrate sequences to determine the genetic relationships. The relationships were estimated based on the pairwise matrix of sequence divergences calculated by ClustalW (Thompson *et al,* 1994). Phylogenetic trees for nucleotide sequences were constructed by the neighbor-joining (NJ) method [Kimura's 2-parameter model, (Kimura, 1980)] using the PHILLIP program. Bootstrap values were tested with 1000 replications for the NJ trees.

Table 5: Genes used in phylogeny

Species	Accession Number
Ovis aries (Sheep) Cytochrome P450 1A1	NM_001129905
Bos taurus (Cattle) Cytochrome P450, family 1,subfamily A (aromatic compound inducible), polypeptide 1(CYP 1A1), mRNA	XM_588298
Sus scrofa (Pig) Cytochrome P450 1A1, (CYP 1A1) Mrna	NM_214412
Equus caballus (Horse) Cytochrome P450, family 1,subfamily A , polypeptide 1(CYP 1A1), mRNA	XM_001493909
Rattus norvegicus (Rat), CYP 3A1	AB008388
Ovis aries (Sheep) Cytochrome P450 subfamily 3A, polypeptide 24 (CYP 3A24)	NM_001129904
Bos taurus (Cattle) Cytochrome P450, subfamily 3A, polypeptide 4 (CYP 3A4)	NM_001099367
Sus scrofa (Pig) Cytochrome P450 3A29, (CYP 3A29) mRNA	NM_214423
Equus caballus (Horse) Cytochrome P450 3A89 (CYP 3A89), mRNA	NM_001101651
Fundulus heteroclitus (Killifish) Cytochrome P450 3A30 (CYP 3A30) mRNA	AF105068
Ovis aries (Sheep), mRNA for metallothionein	X00953
Bos taurus (Cattle), Metallothionein 1E (MT 1E) mRNA	NM_001040492
Sus scrofa (Pig), Metallothionein 1A (MT 1A) mRNA	NM_001001266
Sus scrofa (Boar), mRNA for metallothionein isoform, partial cds	AB000790
Bos grunniens (Yak), Metallothionein 1 (MT 1) mRNA partial cds	AY513744
Homo sapiens (Human) Metallothionein 3 (MT 3), mRNA	NM_005954
Balaena mysticetus (Whale) Metallothionein mRNA, partial cds	AF022117

3.4.6 Quantification of CYP 1A1, CYP 3A and MT 1 mRNAs

Messenger RNA expression levels of CYP 1A1, CYP 3A and MT 1 were measured by quantitative comparative (relative) real-time RT-PCR using DyNamo^{TM} HS SYBR Green qPCR Kit (FINNZYMES, Japan) according to the manufacturer's instructions. A Step one plus real-time PCR system (Applied Biosystems, Japan) was used for PCR amplification and data analysis (step one software version 2.0). The statistical analysis is done in such a way that both the sample and the calibrator data are first normalized against variation in sample quality and quantity. Normalized values, ΔC (t) s, are first calculated from following equations:

$$\Delta C(t)\text{sample} = C(t)\text{target} - C(t)\text{reference}$$

$$\Delta C(t)\text{calibrator} = C(t)\text{target} - C(t)\text{reference}$$

The ΔΔC(t) is then determined using the following formula:

$$\Delta\Delta C(t) = \Delta C(t)\text{sample} - \Delta C(t)\text{calibrator}$$

Expression of the target gene normalized to the reference gene and relative to the calibrator = $2^{-\Delta\Delta C(t)}$. To ensure identical starting conditions, the relative expression data was normalized according to reference (housekeeping) gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The threshold levels on the amplification plots were adjusted to 0.1. It is imperative that the threshold is set correctly to capture the Ct value in the exponential phase of the PCR reaction.

A set of specific primers (SIGMA GENOSYS, Japan) for Kafue lechwe CYP 1A1 and CYP 3A were designed using software "primer 3" (http://frodo.wi.mit.edu/primer3/). We sent the obtained sequences of lechwe CYP 1A1 and CYP 3A to this software which gave us several candidates of primer sets. The primer sets that were used in the experiment are:

- CYP 1A1-F>5'-CAATGGTCTCACCAATGCAC-3'
- CYP 1A1-R>5'-TGAACCAGTGGCAGATCAAC-3'
- CYP 3A- F>5'-CCACGTGTGGACTTTCTTCA-3'
- CYP 3A-R>5'-TGGTCTCATAGCCAGCAAAA-3'

The MT 1 primers used were as follows:

- MT 1-F >5'-ATGGACCCGAACTGCTCCTGC-3'
- MT 1-R>5'- GGCGCAGCAGCTGCACTTGTCCG-3'

These were designed using the Y-MT-SP primers by changing s to g and r to g according to the sequence result of lechwe MT 1 obtained.

The primers used for the reference gene GAPDH were as follows according to Pfister-Genskow *et al*, 2005:

- GAPDH BC102589-F> 5'-GGCGTGAACCACGAGAAGTATAA-3'
- GAPDH BC102589-R> 5'-CCCTCCACGATGCCAAACT-3'

They were designed using Primer Express software (Applied Biosystems, Japan). The melting point peaks for the primers were as follows: CYP 1A1 (around 81.0°C), CYP3A (77.2°C), MT 1 (88.2°C) and GAPDH (83.1°C).

The 2-fold master mix was removed from -20°C freezer and template 25- fold cDNA was removed from -80°C freezer and were thawed on ice. The forward and reverse primers (SIGMA GENOSYS), rox passive reference dye, and DEPC water were removed from -20°C freezer and thawed at room temperature. The individual reagents were mixed to assure homogeneity and then stored on ice immediately. The PCR premix was prepared by mixing 2-fold master mix, primers, rox passive reference dye and DEPC water. The PCR premix was thoroughly mixed to assure homogeneity. Appropriate volumes of 18µl of the PCR premix were dispensed into strip tubes and then template cDNA was added to the strip tubes. Each tube contained 6.8 µl of DEPC water, 10µl of 2-fold master mix, 0.4 µl of 10mM forward primer, 0.4 µl of 10mM reverse primer, 0.4 µl of 50 fold rox passive dye reference and 2 µl of 25 fold cDNA and assumed a final volume of 20µl . The tube strips were gently tapped and then spun down and placed in the real time thermal cycler. Conditions for quantitative real-time RT-PCR were as follows: Holding stage at 95°C for 15 seconds, Cycling stage (35 cycles) at 95°C for 15 seconds and 62°C for 1 minute, Melt curve stage at 95°C for 15 seconds, 60°C for 1 minute and then increased by 0.3°C until 95°C and 95°C for 15 seconds. In our experiment, quadruple sampling was done which provided the ideal number of replicates for each sample. Less than this would have potentially risked an unacceptable high error of measurement, whereas excessive

replication would have resulted in an increase of the cost and reduced the number of samples that could be analyzed. The relative CYP 1A1, CYP 3A and MT 1 mRNA expression levels in each sample were normalized to the housekeeping gene GAPDH cDNA content. Product identity was confirmed by amplicon size with 1.5% agarose /ethidium bromide gel electrophoresis after real-time RT-PCR and melting curve analysis (melting point peak). The expected amplicon sizes were 167bp, 130bp, 183bp and 120bp for CYP 1A1, CYP 3A, MT 1 and GAPDH respectively.

3.5 Data Handling, Storage and Analysis

All laboratory results were stored in excel format for handling and data cleaning. Messenger RNA expression of CYP 1A1, CYP 3A and MT 1 was expressed as mean ± standard error about the mean for each site. Single factor ANOVA was employed to detect the differences in CYP 1A1, CYP 3A and MT1 mRNA expression levels between Blue Lagoon and Lochnivar GMAs. Statistical analysis was performed using JMP 7.0.1 (SAS Institute, Cary, NC, USA). The $P < 0.05$ level was considered significant.

CHAPTER 4.0 RESULTS

4.1 Postmortem Results

The distributions of ecto and endo parasite species and lungs showing TB granulomatous lesions in the 38 sampled Kafue lechwe are shown in Appendix 1.0. Two species of endoparasites (Amphistome and Liver flukes) were clearly identified on visual inspection during postmortem, with two species of ecto parasites (Cutaneous larvae and Ticks).

4.2 Total RNA Quality and Concentration

The concentration and purity of total RNA extracted from liver was determined spectrophotometrically. The 260/280 ratio which is the absorbance obtained at 260nm (wave length) divided by the absorbance obtained at 280nm was used to measure the purity or quality of the RNA. A range of 1.8 to 2.0 entails that RNA is protein free. The 260/280 ratio in this experiment was in the range of 1.85 to 1.99 and the concentration ranged from 158.9 to 1666.4 ng/µl, (Appendix 4.0). Calculations for 1µg RNA required for cDNA Synthesis are shown in Appendix 5.0.

4.3 PCR Amplification

Kafue lechwe CYP 1A1, CYP 3A and MT 1 partial cDNAs were amplified from total liver RNA by gradient RT-PCR. Bands of expected sizes were obtained from the amplified cDNA fragment of CYP 1A1, CYP 3A and MT 1 and were 252bp, 500bp and 183bp following agarose gel electrophoresis as shown in Figure 8 respectively.

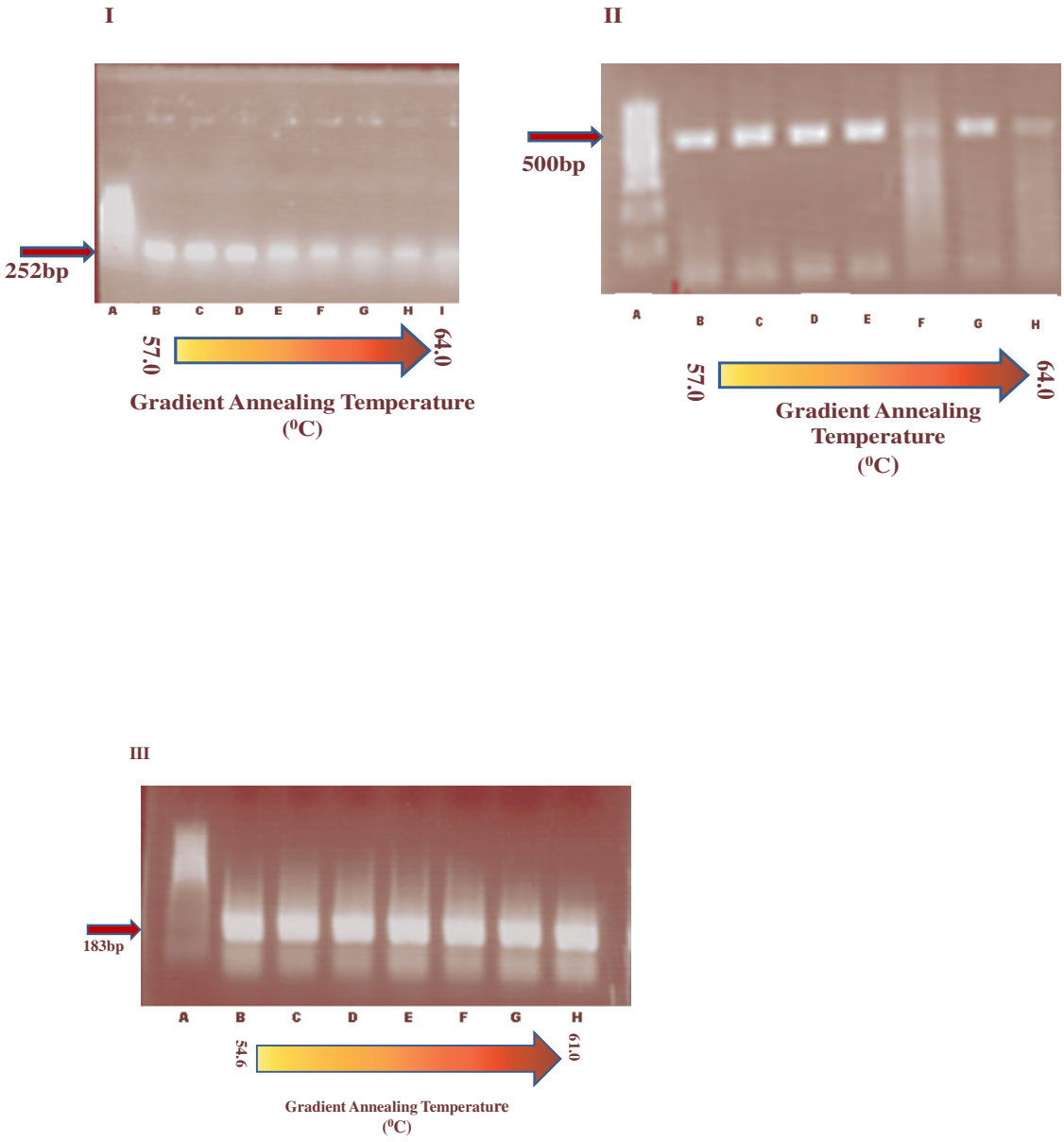

Figure 8: Lechwe CYP 1A1, CYP 3A and MT 1 PCR Product sizes on 1.5% Agarose Gel

I= CYP 1A1, II= CYP 3A, III= MT 1

A= 100bp DNA Ladder

B, C, D, E, F, G and H= Bands of PCR Products

The PCR products with the brightest bands for each locus were chosen for direct sequencing. These were from wells B, C and D in the case of CYP 1A1, wells C, D and E in the case of CYP 3A and from wells B, C, D and E in the case of MT 1.

4.4 Complementary DNA sequences

Upon sequencing of the PCR products, the amplified fragments were confirmed by BLAST search against the NCBI sequence database to be and to contain cDNA encoding CYP 1A1, CYP 3A and MT 1 of Kafue lechwe, with lengths of 206bp, 383bp and 183bp respectively. The results of sequencing of CYP 1A1, CYP 3A and MT 1 are shown below.

- CYP 1A1

CTTTGTGAACCAGTGGCAGATCAACCATGACCAGAAGCTATGGGAGGATCC
ATCTGAGTTCCGGCCAGAACGGTTTCTCACTACTGATGGCACTGTCAACAA
AGTACTGAGTGAGAAGGTGATTATTTTTGGCTTGGGCAAGCGGAAGTGCAT
TGGTGAGACCATTGCCCGCTTGGAGGTCTTTCTCTTCTTGGCCATCCTGCTG
C

- CYP 3A

GAGAATGCCAAGAAGCTCTTAAGATTTGATATCCTCGATCCATTTCTTCTCT
CAGTAGTACTCTTTCCATTCCTTGTCCCAATATTTGAAGTATTAAATATCAC
CATATTTCCCAAAAGTGCTGTGAATTTTTTGACAAAATCTGTAAAAAGGAT
AAAAGAAAGTCGCCTCAAAGATAATCAAAAGCCACGTGTGGACTTTCTTCA
GCTGATGATTAACTCTCAGGATTCCAAAGAAACAGACAATCATAAAGCTCT
CTCTGACCAAGAACTCATGGCCCAAAGTGTTATCTTTATTTTTGCTGGCTAT
GAGACCACTAGCAATACGCTTTCCTTCCTTTTGTATATTTTGGCCACTCACC
CTGATGTCCAGCAGAAGCTGCA

- MT 1

ATGGACCCGAACTGCTCCTGCCCCGCTGGCGGCTCCTGCAGCTGCGCTGGT
TCCTGCACCTGCAAGGCCTGCAGATGCCCCTCCTGCAAGAAGAGCTGCTGC
TCCTGCTGCCCTGTGGGCTGTGCCAAGTGTGCCCAGGGCTGTGTCTGCAAA
GGAGCCTCGGACAAGTGCAGCTGCTGCGCC

4.5 Genetic relationships

The genetic relationships of lechwe CYP 1A1, CYP 3A and MT 1 genes with orthologous genes of other animals were estimated based on the pairwise matrix of sequence divergences calculated by ClustalW and Phylogenetic analysis.

4.5.1 Clustal W Alignment

The deduced partial nucleotide sequences of lechwe CYP 1A1, CYP 3A and MT 1 were compared with those of sheep (*Ovis aries*), cattle (*Bos taurus*), pig (*Sus scrofa*), horse (*Equus caballus*), yak (*Bos grunniens*), and whale (*Balaena mysticetus*) to determine sequence identities by using Clustal W. The alignments are shown in Figures 9, 10 and 11 for CYP 1A1, CYP 3A and MT 1 respectively.

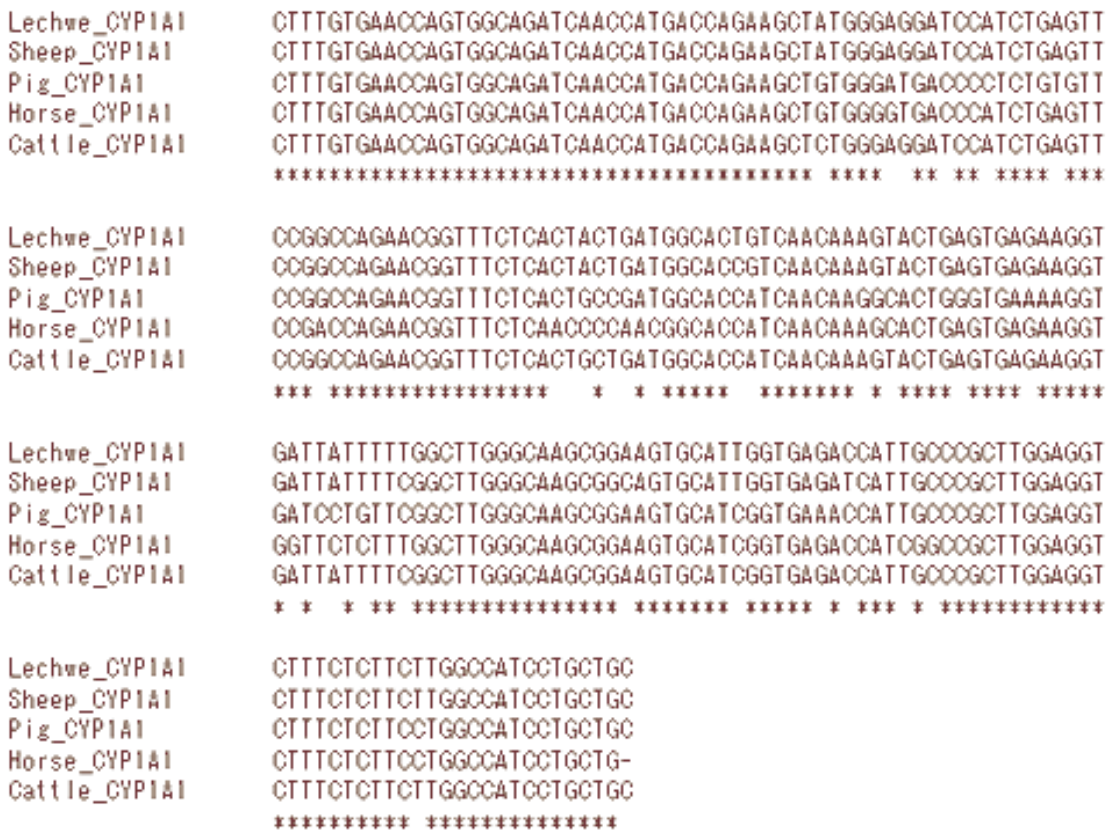

Figure 9: Alignment of CYP 1A1 partial nucleotide sequence of Kafue lechwe, Sheep, Pig, Horse and Cattle

Figure 10: Alignment of CYP 3A partial nucleotide sequence of Kafue lechwe, Sheep, Cattle, Horse and Pig

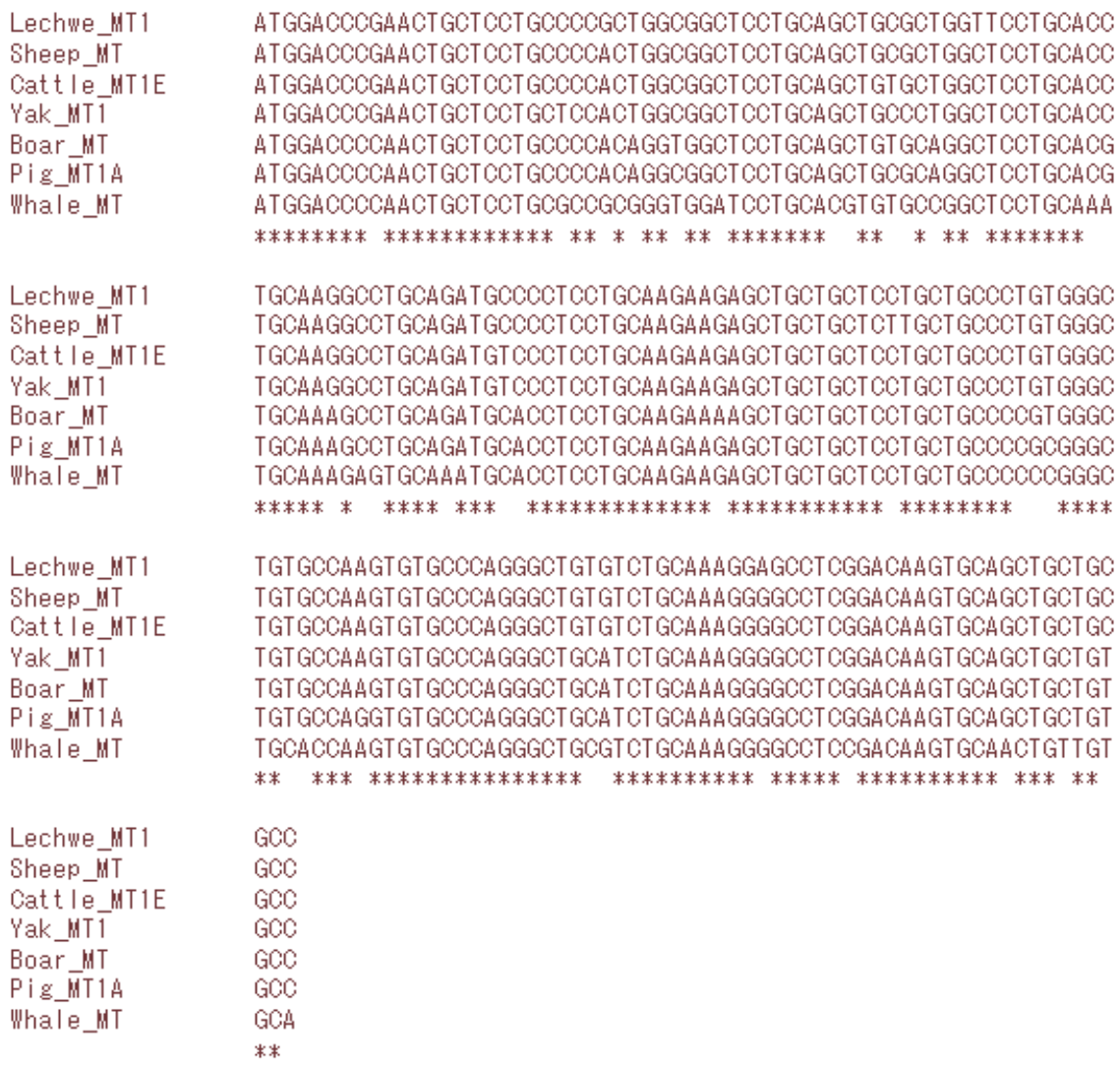

Figure 11: Alignment of MT 1 partial nucleotide sequence of Kafue lechwe, Sheep, Cattle, Yak, Boar, Pig and Whale

*** indicates similarities**

According to the pairwise matrix of sequence divergences calculated by ClustalW the deduced partial nucleotide sequence of Kafue lechwe CYP 1A1 fragment revealed higher identities with those of sheep_ open reading frame (ORF) (98%) and cattle_ORF (97%), followed by that of pig _ORF (90%) and horse_ORF (89%), (Table 6). The deduced partial nucleotide sequence of Kafue lechwe CYP 3A was most closely related to sheep _ORF (97%) and cattle _ORF (95%), but shared lower identities with horse _ORF (84%) and pig

_ORF (83%), (Table 7). That of lechwe MT 1 revealed higher identities with those of sheep _ORF (97%) and cattle _ORF (97%) followed by that of yak _ORF (95%), boar _ORF and pig _ORF (91%) and whale _ORF (84%), (Table 8).

Table 6: Nucleotide identities of the CYP 1A1 for Lechwe, Cattle, Pig and Horse

	Cattle	Pig	Horse	Lechwe
Sheep	97%	90%	88%	98%
Cattle		92%	90%	97%
Pig			90%	90%
Horse				89%

Table 7: Nucleotide identities of the CYP 3A for Lechwe, Cattle, Pig and Horse

	Cattle	Pig	Horse	Lechwe
Sheep	94	84	84	97
Cattle		83	83	95
Pig			82	83
Horse				84

Table 8: Nucleotide identities of the MT 1 for Lechwe, Cattle, Yak, Boar, Pig and Whale

	Cattle	Yak	Boar	Pig	Whale	Lechwe
Sheep	98	96	92	92	84	97
Cattle		96	92	92	84	97
Yak			92	93	84	95
Boar				97	87	91
Pig					97	91
Whale						84

4.5.2 Phylogenetic Analysis

Phylogenetic trees of the nucleotide sequence of lechwe CYP 1A1, CYP 3A and MT 1 and CYP 1A1, CYP 3A and MT 1 of 5 mammalian CYP 1A1s [(sheep (*Ovis aries*), cattle (*Bos taurus*), pig (*Sus scrofa*), horse (*Equus caballus*) and CYP 3A1 of rat (*Rattus norvegicus* used as an out group)] and 4 mammalian CYP 3A[(sheep (*Ovis aries*), cattle (*Bos taurus*), pig (*Sus scrofa*), horse (*Equus caballus*), and 1 fish, Killifish (*Fundulus heteroclitus* used as an out group)] and 7 mammalian MT [(sheep (*Ovis aries*), cattle (*Bos taurus*), domestic pig (*Sus scrofa*), pig (*Sus scrofa*), horse (*Equus caballus*), yak (*Bos grunniens*), human (*Homo sapiens*) and whale (*Balaena mysticetus* used as an out group)] from multiple alignments were constructed the by neighbor-joining (NJ) method and shown with bootstrap values (Figures 12, 13 and 14). Rat CYP 3A1, killifish CYP 3A30 and human MT 3 were used as out-group species in the CYP 1A1, CYP 3A and MT 1 rooted trees respectively. Phylogenetic analysis of mammalian CYP 1A1 subfamily generated a rooted tree and revealed that Kafue lechwe CYP 1A1 belonged to the same group as sheep and cattle. CYP 3A of Kafue lechwe belonged to the same group as sheep CYP 3A24 and cattle CYP 3A4 and a rooted tree was also generated and the phylogenetic

analysis of MT subfamily generated, revealed that Kafue lechwe MT 1 belonged to the same group as sheep MT and cattle MT 1E. The accuracy of the trees was tested using bootstrap values which are indicated at each branch and length of stem of the trees.

Figure 12: Phylogenetic analysis of the mammalian CYP 1A1 based on the multiple alignment of the deduced nucleotide sequences retrieved from the GenBank database. A number at each branch and the length of the stem indicate bootstrap value based on 1000 samplings. Rat CYP 3A1 was used as an out-group species

Figure 13: Phylogenetic analysis of the mammalian CYP 3A based on the multiple alignment of the deduced nucleotide sequences retrieved from the GenBank database. A number at each branch and the length of the stem indicate bootstrap value based on 1000 samplings. Killifish CYP 3A30 was used as an out-group species

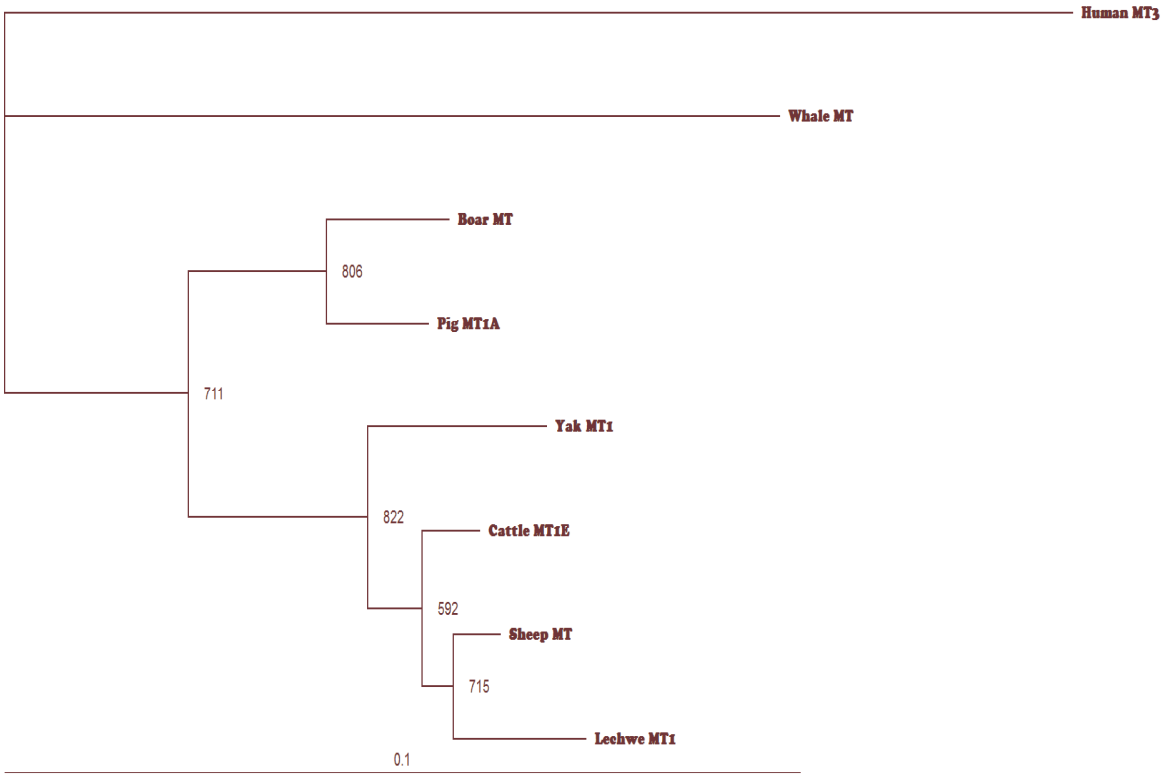

Figure 14: Phylogenetic analysis of the mammalian MT 1 based on the multiple alignment of the deduced nucleotide sequences retrieved from the GenBank database. A number at each branch and the length of the stem indicate bootstrap value based on 1000 samplings. Human MT 3 was used as an out-group species

4.6 Comparative (Relative) Quantification of Kafue Lechwe CYP 1A1, CYP 3A and MT 1 mRNAs

DyNamo^{TM} HS SYBR Green qPCR Kit was used for the relative quantification of CYP 1A1, CYP 3A and MT 1 mRNA expression levels in liver of Kafue lechwe. The final quantification of these target genes was done using the comparative (relative) CT method and is reported as relative transcription or the n-fold difference relative to a calibrator cDNA (i.e., lowest target gene transcription). In brief, the signal of the endogenous control GAPDH was used to normalize the target gene signals of each sample. The relative linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta C(t)}$. Therefore,

all target gene transcription is expressed as an n-fold difference relative to the calibrator. The calibrators for CYP 1A1, CYP 3A and MT 1 were sample number LL07, LL03 and BL01 respectively (Appendices 6.0, 7.0, 8.0). By preliminary examinations of PCR amplification using primers designed for a real-time RT-PCR we confirmed the appearance of single bands with an expected molecular weight of 167bp for CYP 1A1, 130bp for CYP 3A, 183bp for MT 1 and 120bp for GAPDH following agarose gel electrophoresis (Figure 15).

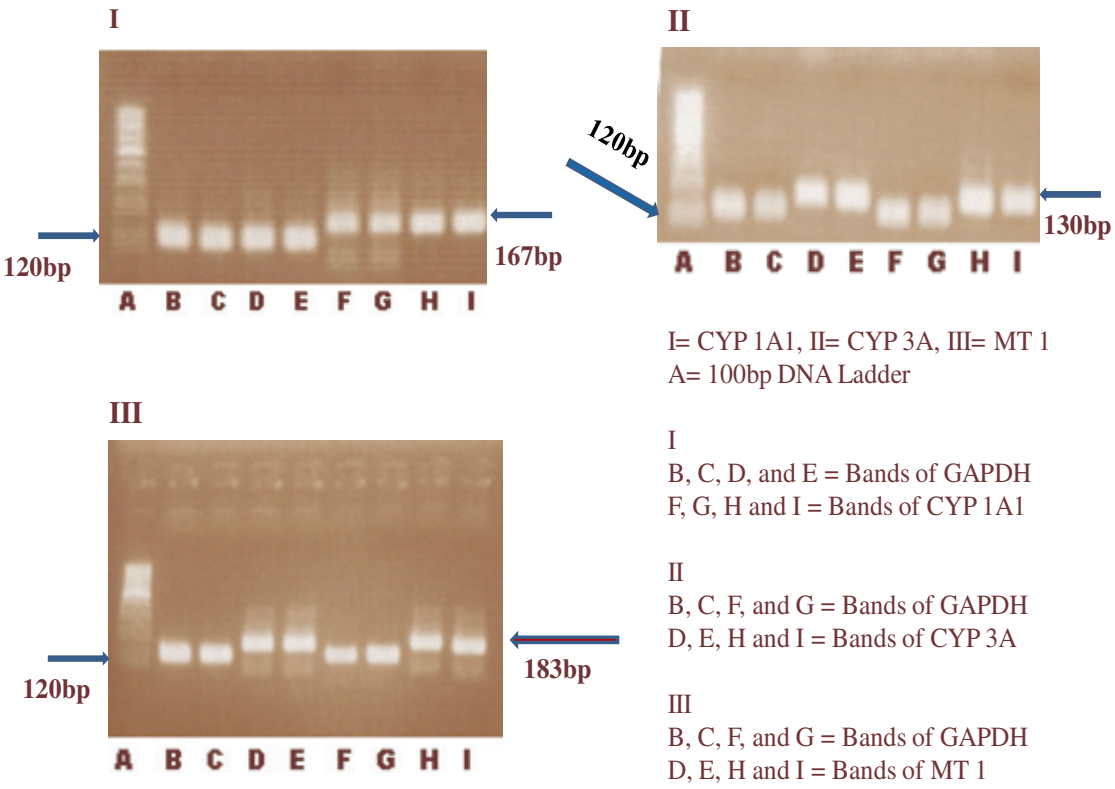

Figure 15: Lechwe GAPDH, CYP 1A1, CYP 3A and MT 1 Real Time PCR Amplicons

Measurement of mRNA expression levels of the genes was done and quantifying mRNA expression levels of CYP 1A1, CYP 3A and MT 1 in the liver samples of Kafue lechwe from both Lochnivar and Blue Lagoon GMAs was a success. Melting curve analysis which was typically included in the analysis software of the real-time fluorescence detection instrument was performed to check the specificity of the amplified products. The principle behind melting curve analysis is that when the temperature is gradually increased, a sharp decrease in SYBR Green fluorescence is observed as the product undergoes denaturation and specific products can be distinguished from the nonspecific products by the difference in their melting temperatures. The melting point peaks for CYP 1A1, CYP 3A and MT 1 were 88.3°C, 77.0°C, 88.44°C and 83.0°C respectively and no sample deviated from these values.

Since quadruple sampling was done in the experiment, the raw data of mean relative quantities for each sample and mean CT values for each sample are presented in appendices 6.0, 7.0 and 8.0 for CYP 1A1, CYP 3A and MT 1 respectively. The mean and standard error about the mean of the data generated is shown in Table 9. From the data it can be observed that there was wide inter-individual variation in gene expression. Therefore, a comparison of the gene expression levels between the two GMAs was determined by normalizing the relative quantities data for Blue Lagoon GMA to one (Appendices 9.0, 10.0 and 11.0). Means and standard error about the mean (SEM) of normalized data were then calculated for CYP 1A1, CYP 3A and MT 1 mRNA expression levels (Table 10).

Table 9: Means and Standard Error about the Mean of CYP 1A1, CYP 3A and MT1 mRNA expression levels

Gene	Lochnivar GMA (n=23)	Blue lagoon GMA (n=15)
CYP 1A1	14.80±4.49	2.42±1.06
CYP 3A	6.34±1.90	5.55±1.58
MT 1	13.09±4.41	25.00±7.48

Table 10: Means and Standard Error about the Mean of CYP 1A1, CYP 3A and MT1 mRNA expression levels for data normalized to 1

Gene	Lochnivar GMA (n=23)	Blue lagoon GMA (n=15)
CYP 1A1	6.12±1.86	1.00±0.44
CYP 3A	1.14±0.34	1.00±0.29
MT 1	0.52±0.18	1.00±0.30

Single factor ANOVA was employed to detect the differences in CYP 1A1, CYP 3A and MT 1 mRNA expression levels between Blue lagoon and Lochnivar GMAs (Tables 11, 12, 13). A 6-fold increase in CYP 1A1 mRNA expression level was seen in Kafue lechwe from Lochnivar GMA when compared with that of Kafue lechwe from Blue Lagoon GMA. The difference was significant ($P < 0.05$). There was no significant difference in CYP 3A and MT 1 mRNA expression levels between the two sites, ($P > 0.05$). Figures 16, 17, and 18 show gene expression levels for CYP 1A1, CYP 3A and MT 1 respectively for the two sites.

Table 11: ANOVA (single factor) for CYP 1A1 expression between Blue Lagoon GMA and Lochnivar GMA

Summary				
Groups	**Count**	**Sum**	**Average**	**Variance**
Blue lagoon GMA	15	15	1	2.88
Lochnivar GMA	23	140.75	6.12	79.32

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	237.96	1	237.96	4.80	**0.04**	4.11
Within Groups	1785.44	36	49.60			
Total	2023.40	37				

Since the *P*-Value is less than 0.05, there is a significant difference.

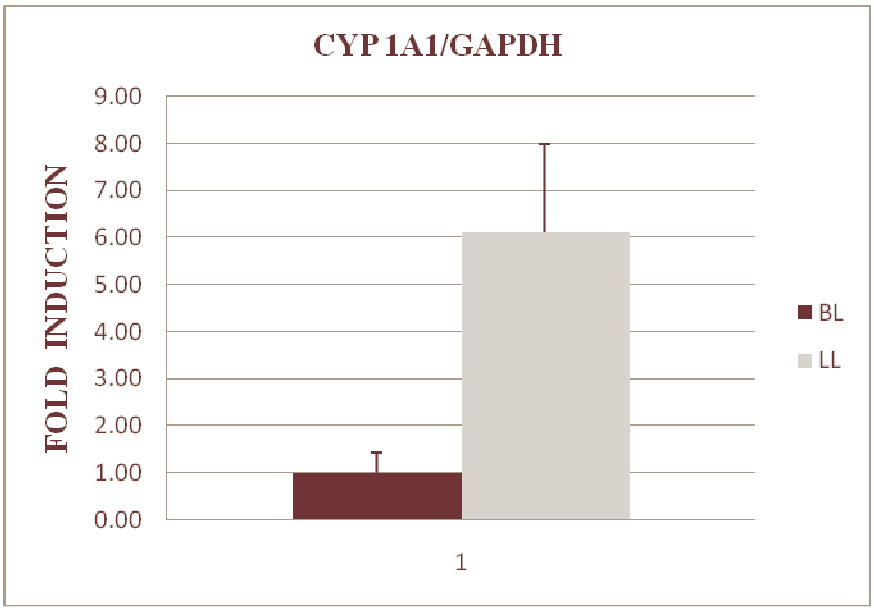

Figure 16: CYP 1A1 gene expression in liver of Kafue lechwe determined by relative RT-PCR

BL= Blue Lagoon GMA, LL= Lochnivar GMA

Table 12: ANOVA (single factor) for CYP 3A expression between Blue Lagoon GMA and Lochnivar GMA

Summary				
Groups	**Count**	**Sum**	**Average**	**Variance**
Blue lagoon GMA	15	15	1.00	1.22
Lochnivar GMA	23	26.28	1.14	2.70

Sources of Variation	**SS**	**df**	**MS**	**F**	**P - value**	**F crit**
Between Groups	0.19	1	0.19	0.09	**0.77**	4.11
Within Groups	76.52	36	2.13			
Total	76.71	37				

Since the *P*-Value is greater than 0.05, there is no significant difference.

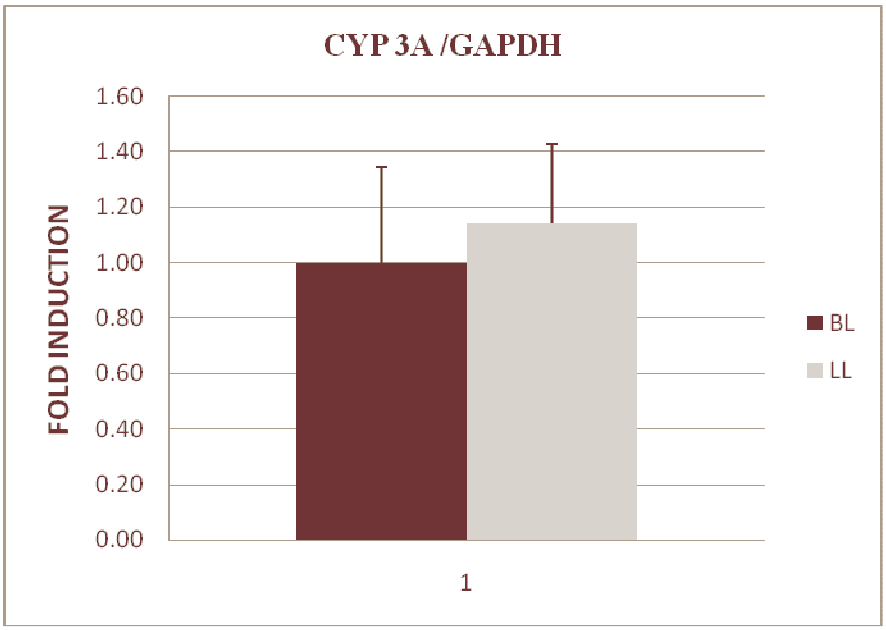

Figure 17: CYP 3A gene expression in liver of Kafue lechwe determined by relative RT-PCR

BL= Blue Lagoon GMA, LL= Lochnivar GMA

Table 13: ANOVA (single factor) for MT 1 expression between Blue Lagoon GMA and Lochnivar GMA

Summary				
Groups	**Count**	**Sum**	**Average**	**Variance**
Blue lagoon GMA	15	15	1	1.34
Lochnivar GMA	23	12.04	0.52	0.72

Source of Variation	**SS**	**df**	**MS**	**F**	**P - value**	**F crit**
Between groups	2.06	1	2.06	2.15	**0.15**	4.11
Within Groups	34.56	36	0.96			
Total	36.62	37				

Since the *P*-Value is greater than 0.05, there is no significant difference.

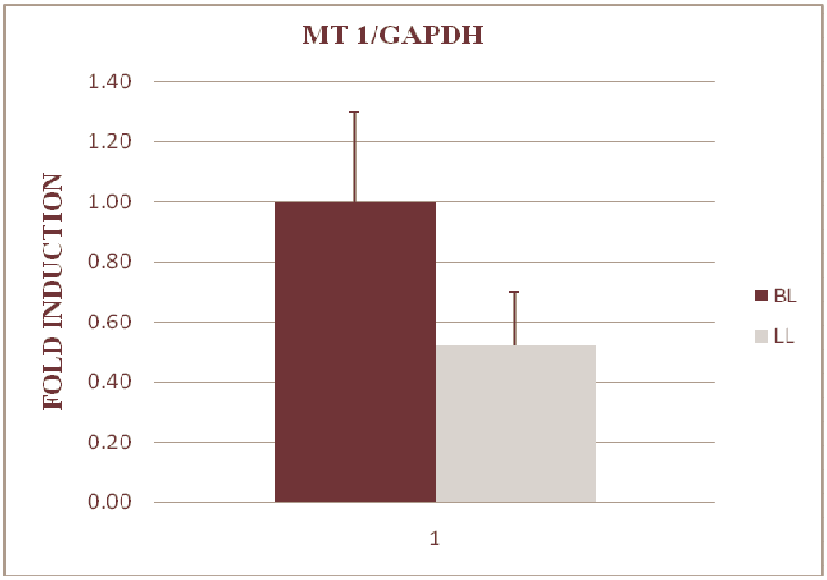

Figure 18: MT 1 gene expression in liver of Kafue lechwe determined by relative RT-PCR

BL= Blue Lagoon GMA, LL= Lochnivar GMA

CHAPTER 5.0 DISCUSSION

5.1 Postmortem Results

Out of the 38 lechwe antelopes sampled, only 8 animals had pathological lesions as indicated in Appendix 1.0.

5.2 Characteristics of CYP 1A1, CYP 3A and MT 1 Partial Nucleotide Sequences

Studies carried out in the waters of the Kafue river have established the presence of several classes of pollutants including pesticides and heavy metals. Accumulation of these contaminants in animals such as the Kafue lechwe that are dependent on the Kafue river has also been reported (Sichilongo and Torto, 2006; Syakalima *et al*, 2001). The presence of these contaminants in the Kafue lechwe can result into known adverse effects that include reproductive and immunological abnormalities as well as numerous pathologies in these animals. Because it is likely that there can be detrimental impact of pollution in Kafue lechwe, knowledge of xenobiotic metabolism in these animals is necessary to precisely evaluate their susceptibility to effects of these chemicals after their exposure to the pollutants in the Kafue river. Although numerous investigations have suggested that expression of CYP 1–4 isoenzymes and MT 1 in animals is altered by xenobiotics including environmental contaminants, and has the potential to be indicative of the exposure to these chemicals (Nebert and Russell, 2002; Hamilton and Merle, 1986), there is no information on the sequence and mRNA expression levels of CYP 450 and MTs genes in Kafue lechwe. Particularly, there is no information on the nucleotide or amino acid sequences of CYP 1A1 which has generally been considered as having attained evolutionary and developmental advantages in defensive responses to xenobiotics. In other animals such as marine mammals, fish, pig eel, the deduced full length and partial nucleotide and/or amino acid sequences of CYP 1A1 have been reported (Niimi *et al*, 2005; Teramitsu *et al*, 2000; Graddy et al, 2000; Aoki *et al*, 1999). Sequencing of CYP 3A genes have been done in other animals such as killifish, whale, seals and chimpanzee, (Celander and Stegeman, 1997; Boon *et al*, 2001; Van Hezik *et al*, 2001; McArthur *et al*, 2003; Williams *et al*, 2004), but neither nucleotide nor amino acid sequence of CYP 3A has been determined in Kafue lechwe. The CYP 3A subfamily in mammals has received

considerable attention over the past ten years, because it has been shown to be induced by therapeutic drugs and other compounds, including natural products, DDT, PCBs and pesticides (Dubois *et al*, 1996; Paolini *et al*, 1996). Furthermore, molecular characterization of MTs has not been done in Kafue lechwe. Metallothioneins are induced by heavy metals and are suitable for assessment of the environmental pollution because of their main physiological functions in scavenging of reactive oxygen species and detoxification of toxic inorganic molecules. Metallothioneins have been cloned in mammals such as mouse, rabbit, sheep, (Durnam *et al*, 1980; Beach and Palmiter, 1981; Searle *et al*, 1984; Stallings *et al*, 1984; Peterson and Mercer, 1986; Peterson *et al*, 1988; Tam *et al*, 1988). Due to the gaps in information about nucleotide and amino acid sequence of CYP 1A1, CYP 3A and MT 1 in Kafue lechwe, we cloned and sequenced these genes and the results provided us with the necessary information for drawing structural/functional inferences as well as insights on the evolution of these gene families. It also provided us with fundamentals for the engineering of these genes that are able to break down specific environmental pollutants and drugs.

This study therefore, determined the presence of CYP 1A1, CYP 3A and MT 1 genes and determined their partial nucleotide sequences in liver of the Kafue lechwe. The genes were sequenced by direct sequencing using an automated DNA sequencer and we successfully managed to isolate and determine the partial nucleotide sequences of CYP lAl, CYP 3A and MT 1 gene open reading frames with lengths of 206bp, 383bp and 183bp respectively. To our knowledge this is the first information on the cDNA sequences of CYP lAl, CYP 3A and MT 1 in liver of Kafue lechwe. By studying the homology of the isolated lechwe CYP 1A1 with CYP 1A1 of other mammals, we found that lechwe CYP 1A1 showed higher similarities with CYP 1A1 of sheep (98%) and cattle (97%). The CYP 3A lechwe sequence shared higher identities with CYP 3A24 of sheep (97%) and CYP 3A4 of cattle (95%) and MT 1 lechwe sequence shared higher identities with MT of sheep (97%) and MT 1E of cattle (97%).

These results therefore strongly suggest that the clones were lechwe CYP 1A1, CYP 3A and MT 1 and could have evolved from the same position in a common ancestral sequence with sheep and cattle. While this study succeeded in identifying CYP 1A1, CYP 3A and

MT 1 genes, other isoenzymes may occur in Kafue lechwe which can be characterized by determining the full length sequences of these genes.

5.3 Phylogenetic Analysis

Phylogenetic analyses of the partial nucleotide sequences were constructed and analyzed by neighbor-joining (NJ) method [Kimura's 2-parameter model, (Kimura, 1980)] using the PHILLIP program. Bootstrap values were tested with 1000 replications for the NJ trees and we confirmed the three novel lechwe genes to be part of the CYP 1A1, CYP 3A and MT 1 subfamilies. Although we managed to construct phylogenetic analyses from the partial nucleotide sequences obtained (i.e. 206 bp, 383bp and 183bp for lechwe CYP 1A1, CYP 3A and MT 1 respectively), there is still a possibility that the phylogeny with full length nucleotide and/or amino acid sequences might be different. However, the phylogenetic analyses carried some reliability because the lechwe clones were located beside sheep and cattle CYP 1A1, CYP 3A and MT 1 suggesting that lechwe, sheep and cattle genes arose from a common ancestor. Moreover, short fragments are useful for the construction of phylogenetic trees (Teramitsu *et al*, 2000). Phylogenetic analysis of mammalian CYP 1A1 revealed that Kafue lechwe CYP 1A1 was clustered in the same group as sheep and cattle. CYP 3A of Kafue lechwe was also clustered in the same group as sheep CYP 3A24 and cattle CYP 3A4 and the phylogenetic analysis of mammalian MT subfamily revealed that Kafue lechwe MT 1 belonged to the same group as sheep MT and cattle MT 1E. These results show that the lechwe genes are most closely related to those of ruminant artiodactyls and hence possibilities exist for similar metabolism of environmental pollutants and drugs, thus, expanding the model systems that may be used for environmental monitoring and new drug development. Although the lechwe genes were solidly grouped in one major cluster of ruminant artiodactyl species, additional CYP 1A1, CYP 3A and MT 1 sequences from other mammals such as cat (or any candidate of the order Carnivora) and marine mammal would more clearly separate the mammalian cluster into two more clusters of carnivore genes and cetacean genes, the orthodox taxonomy in mammalian species (Cao *et al*, 1994; Graur and Higgins, 1994).

The accuracy of the trees was tested using bootstrap values. Bootstrapping essentially tests whether the whole dataset is supporting the tree, or if the tree is just a marginal winner

among many nearly equal alternatives. It is done by taking random subsamples of the dataset, building trees from each of these and calculating the frequency with which the various parts the tree is reproduced in each of these random subsamples (Baldauf, 2003). If group X is found in every subsample tree, its bootstrap support is 100%, if it's found in only two-thirds of the subsample trees; its bootstrap support is 67%. Bootstrap analyses have shown that it is a generally dependable measure of phylogenetic accuracy, and those values of 70% or higher are likely to indicate reliable groupings (Hillis and Bull, 1993). The phylogenetic trees generated in our work showed that lechwe CYP 1A1, CYP 3A and MT 1 belonged to the same group as sheep with bootstrap values of 838 (83.8%), 963 (96.3%) and 715 (71.5%) respectively. The results show that the groupings were reliable.

5.4 Expression levels of CYP 1A1, CYP 3A and MT 1 mRNAs in Liver of Kafue Lechwe

Prior to clinical manifestation, the first interaction between a xenobiotic compound and organism occurs on a molecular level. Thus, if a target molecule of a toxic agent is known, changes in that molecule may be used as a biomarker of exposure or effect and can be measured directly in biological samples. As such the identification and sequencing of CYP lAl, CYP 3A and MT 1 in Kafue lechwe provided us with the invaluable information for the development of potential biomarkers of exposure to and effects of pollutants in these animals. The mRNA expression levels of CYP 1A1, CYP 3A and MT 1 were determined as biomarkers of pollution in liver of Kafue lechwe by reverse transcriptase. This study shows that relative quantitative RT-PCR can be used to detect and quantify CYP lAl, CYP 3A and MT 1 gene expression in liver of Kafue lechwe. The genes were found to be expressed in all the liver samples from both Lochnivar and Blue Lagoon GMAs using this method.

In previous studies, catalytic assays of CYP 1A1 and CYP 3A induction have also proved to be extremely useful tools for assessing exposure to xenobiotics (Burke and Mayer, 1974; Waxman and Chang, 2008). However, the RT-PCR assay has some advantages over the catalytic assays commonly used to measure gene induction (Burke and Mayer, 1974; Klotz *et al*, 1984). Quantitation of mRNA levels is a direct indicator of gene expression, and, as a consequence may not be as subject to interference by endogenous and exogenous

factors that can interfere with enzyme activity. For example, CYP 1Al enzyme activity can be inhibited by common environmental contaminants such as cadmium (Förlin *et al*, 1986; George and Young, 1986) and acrylamide (Haasch *et al*, 1992). In addition, enzyme activity can be modulated by endogenous factors such as steroids (Pajor *et al*, 1990; Goksøyr and Förlin, 1992) and exogenous influences such as temperature (Kloepper-Sams and Stegeman, 1992) and a variety of dietary factors (Ankley *et al*, 1989; Vigano *et al*, 1993; Goksøyr *et al*, 1994). The RT-PCR assay is approximately 1000 times more sensitive than some of the traditional enzyme assays, although enzyme assays are becoming increasingly sensitive and less time consuming (Kennedy and Jones, 1994). The sensitivity of this assay enabled us to quantify mRNA expression levels of CYP lAl, CYP 3A and MT 1 in liver of Kafue lechwe.

This study showed that hepatic CYP 1A1, CYP 3A and MT 1 mRNA was expressed in all the lechwe samples from both Lochnivar and Blue Lagoon GMAs. The mean and standard error about the mean for mRNA levels of CYP 1A1, CYP 3A and MT 1 in lechwe from Lochnivar GMA (n=23) were 14.8 ± 4.49, 6.34 ± 1.90 and 13.09 ± 4.41 respectively. In Blue Lagoon GMA (n=15) the results for the mean and standard error about the mean for mRNA levels of CYP 1A1, CYP 3A and MT 1 were 2.42 ± 1.06, 5.55 ± 1.58 and 25.00 ± 7.48 respectively. From these results, we observed that there was inter-sample/inter-individual variation in the gene expression of all the 3 genes in Kafue lechwe from both sites. We speculate that the probable source of variability could have been in the quantitative RT-PCR experiment itself especially the RT step, as salts, alcohols, or phenol carried over from the RNA isolation can affect the RT efficiency (Freeman *et al*, 1999). Another caveat inherent to any quantitative RT-PCR technique is the difficulty of accounting for inter-samples/inter-individual variability due to potential differences in RT or PCR efficiencies between samples (Wang *et al*, 1989). It has been reported (Suzuki *et al*, 2000) that the inter-sample/inter-individual and inter experiment variability can be resolved or at least minimized by the use of internal controls such as GAPDH and β-actin. To counter the inter-individual variability we used GAPDH, a key enzyme in glycolysis as an internal control and by definition, this gene should have mRNA expressions that are constant under all treatments and experimental conditions. However, there are times that GAPDH cannot be ideal as an internal standard (Oliveira *et al*, 1999; Thellin *et al*, 1999).

GAPDH concentrations vary significantly between different individuals (Bustin *et al*, 1999), during pregnancy (Cale *et al*, 1997), with developmental stage (Puissant *et al*, 1994; Calvo *et al*, 1997), and during the different stages of the cell cycle (Mansur *et al*, 1993). Growth hormone (Freyschuss *et al*, 1994), vitamin D (Desprez *et al*, 1992), oxidative stress (Ito *et al*, 1996), hypoxia (Graven *et al*, 1994; Zhong and Simons, 1999) and manganese (Hazell *et al*, 1999) have all been shown to activate its transcription. Furthermore, it has been shown to be upregulated in cancer – for example in rat hepatomas (Chang *et al*, 1998), malignant murine cell lines (Bhatia *et al*, 1994) and human prostate carcinoma (Ripple and Wilding, 1995). Conversely, food deprivation (Yamada *et al*, 1997) and retinoic acid (Barroso *et al*, 1999) down regulate GAPDH transcription in the gut and in adipocytes, respectively. Finally, numerous transcription regulatory domains have been identified in the yeast GAPDH promoter, again suggesting that this gene is subject to complex transcriptional regulation (Yagi *et al*, 1994). It is thus important that in this experiment more than one endogenous internal control e.g. GAPDH and β-actin should have been used simultaneously in order to define and correct the possible inter-individual variation. This combined approach has been previously reported with GAPDH and β-actin as the two internal controls (Zhong and Simons 1999). Alternatively, homologous synthetic RNA standards instead of two endogenous internal controls could have been used. This type of standard involves a synthetic RNA sharing identical primer binding sites with the native RNA and the same internal sequence except for a short deletion or insertion (Wang *et al*, 1989; Freeman *et al*, 1999). Homologous synthetic RNA standards are most likely to have the same RT and PCR efficiency as their native counterparts but their creation and use are more complex than that of endogenous standards (Zimmermann and Mannhalter 1996).

Other possible sources of variation especially for CYP 1A1 could be age and size, reproductive stage and sex hormones of the Kafue lechwe. Sex hormones are among factors that influence expression of CYP 1A1. During sexual maturation, a higher content of CYP 1A1 is normally present in males than females (Larsen *et al*, 1992; Stegeman and Hahn, 1994). Oestrogenic compounds such as 17β-estradiol are known as down regulators of CYP 450s (Elskus *et al*, 1992). The influence of size is logical from the fact that the amount of pollutants taken may differ depending on the size of the animal. The main age

related factors influencing expression of CYP 1A1 is the length of time the animal has had to accumulate the inducing contaminants and the reproductive stage of the organism. This has been observed in adult fish that have accumulated CYP 1A1 inducing contaminants compared to juveniles (Whyte *et al*, 2000). Age and sex are also possible sources of Inter individual variation of gene expression of CYP 3A in Kafue lechwe. Sex and age represent some constitutional factors that are likely to contribute to the modulation of the overall biotransformation capacity of CYP 3A (Maurel, 1996; Nebbia, 2001). It is known that age and sex might contribute to the expression and regulation of hepatic CYP 3A in man, laboratory species and domestic animals (Nebbia, 2001; Guengerich, 2002). Since 1932, sex-related differences in the way by which animals metabolize xenobiotics and drugs have been reported (Harris *et al*, 1995); in this respect, a clear sexual dimorphism in the expression of hepatic genes, including some members of the CYP 3A subfamily, has been documented in rodents and, particularly, in rats (Anakk *et al*, 2003; Furukawa *et al*, 1999; Kato and Yamazoe, 1992). Moreover, unlike males, females have depuration associated with reproduction and lactation and contaminant concentrations decline throughout the reproductive years. This has been seen in cetaceans (Aguilar and Borrell 1988). Genetic polymorphism is also one of the factors resulting in variation of CYP 450 expression and gives rise to distinct subgroups in the population that differ in their ability to perform certain biotransformation reactions (Van der Weide and Steijns, 1999).

The extent of MT induction can also vary depending on diet and nutrition, reproductive status and tissue regeneration (Bucheli and Fent, 1995). All these factors need to be taken into account when MT is used as a biomarker. The factors mentioned here therefore, could have caused inter- individual variations in gene expression of CYP 1A1, CYP 3A and MT 1 in Kafue lechwe and maybe potential sources of error.

Due to inter- individual variations in gene expression in all the 3 genes, we normalized the Blue Lagoon relative quantities data to one (n=15) and single factor ANOVA was employed to analyze significant differences for CYP 1A1, CYP 3A and MT 1 mRNA levels between Lochnivar and Blue Lagoon GMA. The mean and standard error about the mean (SEM) of normalized data for CYP lAl, CYP 3A and MT 1 mRNA expression levels in lechwe from Lochnivar GMA (n=23) was 6.12 ± 1.86, 1.14 ± 0.34 and 0.52 ± 0.18

respectively. There was a 6 fold increase in CYP 1A1 mRNA expression in lechwe from Lochnivar GMA compared to lechwe from Blue Lagoon GMA and the difference was significant ($P<0.05$). The difference could have been attributed to the fact that unlike Blue Lagoon GMA, Lochinvar used to be a private ranch between 1908 and 1965 (Rottcher, 1998) a period that brackets the era when organochlorine (OC) pesticides were first synthesized and put to use for cattle dipping and other activities meant to annihilate pests in agriculture and domestic households. The Lochinvar ranch gained National Park status on the 25th of February 1972. Even during the time it was a ranch up to today, it has not been spared in efforts to eradicate undesirable pests and weeds by agricultural activists. Therefore it is possible that the concentration of CYP 1A1 inducers in Lochnivar GMA is higher than Blue Lagoon GMA thus the significant difference. Feeding stress could be another factor that could have led to the significance difference in CYP 1A1 mRNA expression levels. Usually Blue Lagoon GMA is flooded throughout the year and hence forage is scarce for the animals. As such contaminant intake through foraging is minimal. Therefore, it is possible that differences in contaminant concentrations (CYP 1A1 inducers) may account for the regional differences in CYP 1A1 mRNA expression. It is therefore important that plant and soil samples be collected from both Lochnivar and Blue Lagoon GMAs to analyze for organic chemicals and correlate the results with the gene expression levels in order for us to come up with definite conclusions. There was no significance difference in CYP 3A and MT 1 mRNA expression levels ($P>0.05$) between Blue Lagoon GMA and Lochnivar GMA. The possible reasons could be that the distribution of CYP 3A and MT 1 inducers in the two sites could be the same.

CHAPTER 6.0 SUMMARY AND CONCLUSIONS

6.1 Identification and Expression of CYP 1A1, CYP 3A and MT 1 in Liver of Kafue Lechwe

This study assessed the potential of using CYP 1A1, CYP 3A and MT 1 as biomarkers of contamination of the Kafue lechwe in the Kafue river. The study successfully isolated partial genes of CYP 1A1, CYP 3A and MT 1 in liver of Kafue lechwe and the deduced partial nucleotide sequences were open reading frames of cDNA encoding CYP 1A1, CYP 3A and MT 1 of Kafue lechwe, with lengths of 206bp, 383bp and 183bp respectively. This study represents the first report on isolation and characterization of these enzymes in Kafue lechwe. Furthermore, according to the pairwise matrix calculated by Clustal W, the cDNA nucleotide sequence of lechwe CYP 1A1 fragment showed higher identities with those of sheep CYP 1A1 (98%) and cattle CYP 1A1(97%). The deduced partial nucleotide sequence of Kafue lechwe CYP 3A was closely related to sheep (97%) and cattle (95%). That of lechwe MT 1 revealed higher identities with those of sheep (97%) and cattle (97%). Subsequently, it was possible to make phylogenetic analysis of mammalian CYP 1A1, CYP 3A and MT 1. The mammalian CYP 1A1 tree showed that lechwe CYP 1A1 was clustered in the same group as that of sheep and cattle CYP 1A1. The mammalian CYP 3A tree showed that lechwe CYP 3A was in the same group as that of sheep CYP 3A24 and cattle CYP 3A4. Phylogenetic analysis of MT subfamily revealed that Kafue lechwe MT 1 belonged to the same group as sheep MT and cattle MT 1E. The phylogenetic trees generated were tested by bootstrapping and it was shown that lechwe CYP 1A1, CYP 3A and MT 1 belonged to the same group as sheep with bootstrap values of 838 (83.8%), 963 (96.3%) and 707 (70.7%) respectively and the results show that the groupings were reliable. The results of identification and characterization of lechwe CYP 1A1, CYP 3A and MT 1 show that the lechwe genes are most closely related to those of ruminant artiodactyls and hence possibilities exist for similar metabolism of environmental pollutants. Identification of CYP 1A1, CYP 3A and MT 1 genes in Kafue lechwe that are exposed to high risks of environmental pollution provided us with new insights into the metabolic or toxicological functions of these genes in these animals and based on the

results, we determined the mRNA expression levels of CYP 1A1, CYP 3A and MT 1 as biomarkers of pollution in liver of Kafue lechwe by relative quantitative real time RT-PCR. The study showed that hepatic CYP 1A1, CYP 3A and MT 1 mRNA levels were expressed in all the lechwe liver samples from both Lochnivar and Blue Lagoon GMAs. There was a 6 fold increase in CYP 1A1 mRNA expression in lechwe from Lochnivar GMA compared to lechwe from Blue Lagoon GMA and the difference was significant ($P<0.05$). However there was there was no significance difference in CYP 3A and MT 1 mRNA expression levels ($P>0.05$) between the two sites. This study therefore demonstrates that hepatic CYP 1A1, CYP 3A and MT 1 mRNA expression levels have the potential to be used as a biomarkers of exposure and effect to pollution in Kafue lechwe.

In future, however, characterization of CYP 1A1, CYP 3A and MT 1, such as measurement of tissue distribution of mRNA expression, determination of full-length sequence of nucleotides and amino acids and characterization of enzyme activities in Kafue lechwe will be determined to complement these results . Substrate specificity and inducibility of the new CYP 1A1, CYP 3A and MT 1 genes we identified in Kafue lechwe need to be determined before we can assess the overall significance of the CYP 1A1, CYP 3A and MT 1 subfamilies in environmental toxicology with certainty. The influence of age, sex and nutrition on the expression levels of CYP 1A1, CYP 3A and MT 1 genes in Kafue lechwe also need to be explored.

6.2 Significance of the Study

CYP 1A1 induction is currently used as the biomarker of choice for assessing exposure to PAHs and PHAHs, CYP 3A is used as a biomarker for assessing exposure to DDT and other pesticides and MT 1 has been used as a biomarker for assessing heavy metal exposure in a large number of species and in various taxa. This study has established the existence of CYP 1A1, CYP 3A and MT 1 genes and quantified these genes in Kafue lechwe. It is therefore possible that the Kafue lechwe is exposed to these organic and inorganic pollutants originating from the Kafue river and that these pollutants are eliciting biological effects. The study has also provided baseline information concerning pollution in the Kafue lechwe which is essential in bio-monitoring and environmental protection of the Kafue river basin ecosystem. The Kafue lechwe has got public health significance and

therefore the results obtained can be used by public health personnel to assess the safety of the meat harvested from the Kafue lechwe and other wildlife species that are consumed by man from the Kafue river basin ecosystem. The data can also be used by policy makers responsible for environmental pollution and monitoring and wildlife conservation.

6.3 Contributions of the Thesis Research

- Establishment of the existence of CYP 1A1, CYP 3A and MT 1 genes in liver of Kafue lechwe
- Design of CYP 1A1 primers that successfully recognized CYP 1A1 genes in Kafue lechwe
- Identification of CYP 1A1, CYP 3A and MT 1 in liver of Kafue lechwe. This is the first report on these genes in Kafue lechwe
- Cloning of CYP 1A1, CYP 3A and MT 1 in Kafue lechwe. The sequences we obtained provided us with information that is crucial in the identification of the full length sequences
- Design of quantitative RT-PCR for CYP 1A1, CYP 3A and MT 1 mRNAs in liver of Kafue lechwe

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APPENDICES

Appendix 1.0: Results of Visual 1nspection and Postmortem Examination of Lechwe

Sample Name	Amphistome	Liver Flukes	Cutaneous Larvae	Ticks	TB Lesions
LL07	-	-	-	-	-
LL17	-	-	-	-	-
LL31	+	-	-	-	-
LL41	+	+	-	-	-
LL47	-	-	-	-	-
LL11	-	-	-	-	-
LL22	-	-	-	-	-
LL33	-	-	-	-	-
LL44A	-	-	-	-	-
LL49	-	-	-	+	-
LL12	-	-	-	-	-
LL26	-	-	-	-	-
LL38	-	-	-	-	-
LL44B	-	-	-	-	-
LL48	-	-	-	-	-
LL13	-	-	-	-	-
LL28	-	-	-	-	-
LL40	-	-	-	-	+
LL50	-	-	-	-	-
LL45	-	-	-	-	-
LL03	-	-	-	-	-
LL20	-	-	-	-	-
LL21	-	-	-	-	-
BL04	+	+	-	-	-
BL12	-	-	-	+	+
BL22	-	-	-	-	-
BL01	-	-	-	-	-
BL06	-	-	-	-	-
BL15	-	-	-	-	-
BL23	-	-	-	-	-
BL02	-	-	-	-	+
BL08	-	-	-	-	-
BL17	-	-	-	-	-
BL24	-	-	+	-	-
BL03	-	-	-	-	-
BL09	-	-	-	-	-
BL18	-	-	-	-	-
BL25	-	-	-	-	-

Appendix 2.0: 1.5% Agarose Gel/Ethidium Bromide Preparation

a) 7.50g of agarose was weighed and transferred to a clean 1000ml conical flask
b) 500mls of 5X TBE buffer was added and shaken to mix
c) The mixture was then taken to the microwave to dissolve
d) The solution was cooled and to it, 1µl of ethidium bromide was added and mixed well
e) The solution was then poured on plates and allowed to cool until solidification of the gel
f) Then wells were punched into the gels
g) The gels were removed and stored in 0.5X TBE buffer until use

Appendix 3.0: TBE Buffer Preparation (500mls)

a) 30.27g of tris base was weighed and added to a clean 1000ml conical flask
b) 15.00g of boric acid was then added to the flask
c) 20mls of 0.5M EDTA (pH 8.0) was later added and DEPC water was added to make up to 500mls

Appendix 4.0: RNA Concentration and 260/280 Ratio

Blue Lagoon GMA			Lochnivar GMA		
Sample Name	**Concentration ng/µl**	**260/280 Ratio**	**Sample Name**	**Concentration ng/µl**	**260/280 Ratio**
BL03	643.6	1.96	LL07	405.9	1.91
BL04	897.8	1.94	LL11	393.3	1.87
BL06	621.3	1.91	LL12	158.9	1.91
BL17	779.4	1.98	LL13	617.3	1.96
BL24	661.3	1.94	LL17	385.3	1.88
BL25	540.9	1.93	LL20	739.3	1.98
BL01	620.8	1.93	LL21	328.1	1.92
BL02	508.0	1.99	LL26	423.0	1.91
BL08	1666.4	1.94	LL22	539.9	1.94
BL09	817.4	1.98	LL33	869.4	1.96
BL12	910.2	1.98	LL40	572.6	1.85
BL15	466.9	1.89	LL41	953.2	1.96
BL18	656.3	1.96	LL44A	411.6	1.87
BL22	329.2	1.88	LL44B	521.3	1.97
BL23	888.5	1.98	LL47	679.3	1.94
			LL48	705.7	1.98
			LL49	463.8	1.91
			LL50	934.4	1.96
			LL31	958.5	1.96
			LL38	529.6	1.93
			LL45	515.6	1.85
			LL28	303.0	1.95
			LL03	559.4	1.99

Appendix 5.0: Calculations for 1µg RNA required for cDNA Synthesis

Sample Name	RNA Conc. ng/µl	Multiplication Factor	RNA Conc. µg/µl	RNA Conc. Required µg/µl	Vol. of RNA (ng/µl) Obtained µl	Vol. of DEPC water added µl	Total Vol. µl
LL07	405.9	0.001	0.4059	1	2.5	4.0	6.5
LL11	393.3	0.001	0.3933	1	2.5	4.0	6.5
LL12	158.9	0.001	0.1589	1	6.3	0.2	6.5
LL13	617.3	0.001	0.6173	1	1.6	4.9	6.5
LL17	385.3	0.001	0.3853	1	2.6	3.9	6.5
LL20	739.3	0.001	0.7393	1	1.4	5.1	6.5
LL21	328.1	0.001	0.3281	1	3.0	3.5	6.5
LL26	423.0	0.001	0.423	1	2.4	4.1	6.5
LL22	539.9	0.001	0.5399	1	1.9	4.6	6.5
LL33	869.4	0.001	0.8694	1	1.2	5.3	6.5
LL40	572.6	0.001	0.5726	1	1.7	4.8	6.5
LL41	953.2	0.001	0.9532	1	1.0	5.5	6.5
LL44A	411.6	0.001	0.4116	1	2.4	4.1	6.5
LL44B	521.3	0.001	0.5213	1	1.9	4.6	6.5
LL47	679.3	0.001	0.6793	1	1.5	5.0	6.5
LL48	705.7	0.001	0.7057	1	1.4	5.1	6.5
LL49	463.8	0.001	0.4638	1	2.2	4.3	6.5
LL50	934.4	0.001	0.9344	1	1.1	5.4	6.5
LL31	958.5	0.001	0.9585	1	1.0	5.5	6.5
LL38	529.6	0.001	0.5296	1	1.9	4.6	6.5
LL45	515.6	0.001	0.5156	1	1.9	4.6	6.5
LL28	303.0	0.001	0.303	1	3.3	3.2	6.5
LL03	559.4	0.001	0.5594	1	1.8	4.7	6.5
BL03	643.6	0.001	0.6436	1	1.6	4.9	6.5
BL04	897.8	0.001	0.8978	1	1.1	5.4	6.5
BL06	621.3	0.001	0.6213	1	1.6	4.9	6.5
BL17	779.4	0.001	0.7794	1	1.3	5.2	6.5
BL24	661.3	0.001	0.6613	1	1.5	5.0	6.5
BL25	540.9	0.001	0.5409	1	1.8	4.7	6.5
BL01	620.8	0.001	0.6208	1	1.6	4.9	6.5
BL02	508.0	0.001	0.508	1	2.0	4.5	6.5
BL08	1666.4	0.001	1.6664	1	0.6	5.9	6.5
BL09	817.4	0.001	0.8174	1	1.2	5.3	6.5
BL12	910.2	0.001	0.9102	1	1.1	5.4	6.5
BL15	466.9	0.001	0.4669	1	2.1	4.4	6.5
BL18	656.3	0.001	0.6563	1	1.5	5.0	6.5
BL22	329.2	0.001	0.3292	1	3.0	3.5	6.5
BL23	888.5	0.001	0.8885	1	1.1	5.4	6.5

Appendix 6.0: Mean Relative Quantities for CYP 1A1 and Mean CT values of each Sample

Blue Lagoon GMA			Lochnivar GMA		
Sample Name	**Mean RQ CYP 1A1**	**CT Mean**	**Sample Name**	**Mean RQ CYP 1A1**	**CT Mean**
BL04	2.0259	27.0443	LL07	1.0000	28.3019
BL12	1.5980	27.5454	LL17	8.5624	23.8210
BL22	0.6065	28.5145	LL31	0.8508	28.0517
BL01	0.3666	29.6150	LL41	0.7565	29.9246
BL06	0.3684	30.0519	LL47	62.7489	21.5902
BL15	0.9036	28.8851	LL11	0.9632	29.1105
BL23	16.7495	24.7867	LL22	1.6762	28.1163
BL02	0.4859	29.4936	LL33	0.1039	30.8982
BL08	3.2562	26.1401	LL44A	1.2377	29.3923
BL17	2.1155	27.1181	LL49	47.1100	22.2278
BL24	2.0368	27.0454	LL12	46.1018	23.4160
BL03	0.8153	28.8538	LL26	12.7525	25.1159
BL09	0.4197	29.2159	LL38	0.2793	31.0491
BL18	0.6969	29.2542	LL44B	0.6920	30.4491
BL25	3.8424	27.3775	LL48	23.2228	24.7751
Mean	**2.4192**		LL13	6.6552	25.9265
SEM	**1.06**		LL28	72.3635	20.5468
			LL40	4.9813	26.6891
			LL50	2.8049	28.4633
			LL45	0.5838	28.3561
			LL03	9.4498	25.7344
			LL20	9.1105	26.4616
			LL21	26.4905	23.4910
			Mean	**14.8043**	
			SEM	**4.49**	

Appendix 7.0: Mean Relative Quantities for CYP 3A and Mean CT values of each Sample

Blue Lagoon GMA			Lochnivar GMA		
Sample Name	**Mean RQ CYP 3A**	**CT Mean**	**Sample Name**	**Mean RQ CYP 3A**	**CT Mean**
BL02	1.8694	29.9459	LL03	1.0000	30.8845
BL03	2.0559	29.9680	LL07	3.6792	27.9276
BL06	1.4556	30.5924	LL11	1.0479	29.9367
BL09	1.7590	30.0627	LL12	23.2362	24.9182
BL12	21.2854	26.2390	LL13	30.9820	25.7596
BL15	2.4804	29.4684	LL17	20.9886	25.5784
BL22	5.1570	28.7395	LL31	5.2362	28.6478
BL23	14.4388	26.6920	LL33	0.2183	30.5478
BL24	4.8199	28.3178	LL38	0.2025	32.5530
BL25	9.2582	27.5265	LL40	1.5821	29.7413
BL01	1.3688	30.4948	LL41	0.1483	30.4620
BL04	1.0296	30.8133	LL44A	0.5072	27.9637
BL08	12.7095	27.1617	LL44B	0.5834	29.4561
BL17	2.7029	28.6986	LL45	1.7396	29.9200
BL18	0.8579	31.4032	LL47	1.5964	26.6277
Mean	**5.5499**		LL48	23.3651	24.4338
SEM	**1.58**		LL20	0.6772	29.6139
			LL21	10.8862	27.4674
			LL22	3.3441	28.1655
			LL26	1.3908	27.7781
			LL28	7.2743	28.3294
			LL49	5.6699	24.7020
			LL50	0.5212	29.7652
			Mean	**6.3425**	
			SEM	**1.90**	

Appendix 8.0: Mean Relative Quantities for MT 1 and Mean CT values of each Sample

Blue Lagoon GMA			Lochnivar GMA		
Sample Name	**Mean RQ MT 1**	**CT Mean**	**Sample Name**	**Mean RQ MT 1**	**CT Mean**
BL01	1.0000	26.1396	LL07	13.3872	23.1711
BL06	1.5697	23.9396	LL17	15.7217	23.8386
BL24	4.8545	23.7966	LL20	4.5359	24.1891
BL02	5.6888	24.0964	LL26	0.7553	25.9935
BL08	6.6266	24.3113	LL31	15.6231	25.2198
BL25	51.0024	20.2638	LL33	0.0459	30.0578
BL03	5.5708	24.6090	LL12	50.8907	19.8071
BL22	29.9888	21.5166	LL13	13.9744	23.2107
BL04	15.0729	22.6658	LL03	10.8837	23.1757
BL23	34.2116	21.3138	LL21	87.6596	19.1015
BL09	1.4455	27.6649	LL11	0.3434	28.7603
BL12	99.0989	20.1514	LL38	0.2717	28.3427
BL15	69.9622	21.7461	LL45	0.1310	30.8391
BL17	33.6796	22.8765	LL48	3.7672	25.2400
BL18	15.2630	24.4545	LL44A	0.2666	27.6460
Mean	**25.0023**		LL50	0.7326	28.7479
SEM	**7.48**		LL44B	0.0678	30.0297
			LL49	2.7560	25.3037
			LL40	0.5493	27.9976
			LL41	0.2547	28.9075
			LL47	38.1856	20.4759
			LL22	9.3979	24.0854
			LL28	30.8855	22.9501
			Mean	**13.0907**	
			SEM	**4.41**	

Appendix 9.0: Mean Relative Quantities for CYP 1A1 of each Sample after Normalization of Blue Lagoon data to 1

Blue Lagoon GMA		Lochnivar GMA	
Sample Name	**Mean RQ CYP 1A1**	**Sample Name**	**Mean RQ CYP 1A1**
BL04	0.84	LL07	0.41
BL12	0.66	LL17	3.54
BL22	0.25	LL31	0.35
BL01	0.15	LL41	0.31
BL06	0.15	LL47	25.94
BL15	0.37	LL11	0.40
BL23	6.92	LL22	0.69
BL02	0.20	LL33	0.04
BL08	1.35	LL44A	0.51
BL17	0.87	LL49	19.47
BL24	0.84	LL12	19.06
BL03	0.34	LL26	5.27
BL09	0.17	LL38	0.12
BL18	0.29	LL44B	0.29
BL25	1.59	LL48	9.60
Mean	**1.00**	LL13	2.75
SEM	**0.44**	LL28	29.91
		LL40	2.06
		LL50	1.16
		LL45	0.24
		LL03	3.91
		LL20	3.77
		LL21	10.95
		Mean	**6.12**
		SEM	**1.86**

Appendix 10.0: Mean Relative Quantities for CYP 3A of each Sample after Normalization of Blue Lagoon data to 1

Blue Lagoon GMA		Lochnivar GMA	
Sample Name	**Mean RQ CYP 3A**	**Sample Name**	**Mean RQ CYP 3A**
BL02	0.34	LL03	0.18
BL03	0.37	LL07	0.66
BL06	0.26	LL11	0.19
BL09	0.32	LL12	4.19
BL12	3.83	LL13	5.58
BL15	0.45	LL17	3.78
BL22	0.93	LL31	0.94
BL23	2.60	LL33	0.04
BL24	0.87	LL38	0.04
BL25	1.67	LL40	0.29
BL01	0.25	LL41	0.03
BL04	0.19	LL44A	0.09
BL08	2.29	LL44B	0.11
BL17	0.49	LL45	0.31
BL18	0.15	LL47	0.29
Mean	**1.00**	LL48	4.21
SEM	**0.29**	LL20	0.12
		LL21	1.96
		LL22	0.60
		LL26	0.25
		LL28	1.31
		LL49	1.02
		LL50	0.09
		Mean	**1.14**
		SEM	**0.34**

Appendix 11.0: Mean Relative Quantities for MT 1 of each Sample after Normalization of Blue Lagoon data to 1

Blue Lagoon GMA		Lochnivar GMA	
Sample Name	**Mean RQ MT 1**	**Sample Name**	**Mean RQ MT 1**
BL01	0.04	LL07	0.54
BL06	0.06	LL17	0.63
BL24	0.19	LL20	0.18
BL02	0.23	LL26	0.03
BL08	0.27	LL31	0.62
BL25	2.04	LL33	0.00
BL03	0.22	LL12	2.04
BL22	1.20	LL13	0.56
BL04	0.60	LL03	0.44
BL23	1.37	LL21	3.51
BL09	0.06	LL11	0.01
BL12	3.96	LL38	0.01
BL15	2.80	LL45	0.01
BL17	1.35	LL48	0.15
BL18	0.61	LL44A	0.01
Mean	**1.00**	LL50	0.03
SEM	**0.30**	LL44B	0.00
		LL49	0.11
		LL40	0.02
		LL41	0.01
		LL47	1.53
		LL22	0.38
		LL28	1.24
		Mean	**0.52**
		SEM	**0.18**

LL: Liver, Kafue lechwe from Lochnivar GMA

BL: Liver, Kafue lehwe from Blue Lagoon GMA