The metabolic profiling of cheetahs (*Acinonyx jubatus*): A systems biology approach to understanding the chronic diseases they suffer in captivity

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DECLARATION

I hereby declare that this thesis contains my own original research and interpretation that has not been submitted for a degree at any other institution.

Adrian S.W. Tordiffe

May 2017

DEDICATION

This work is dedicated to the cheetahs that I have worked with over the years. They are truly unique and magnificent animals.

"Those who dwell, as scientists or laymen, among the beauties and mysteries of the earth, are never alone or weary of life" – Rachel Carson

PREFACE

Motivation for the study

Despite obvious improvements in husbandry conditions in zoos and other captive facilities around the world, cheetahs continue to suffer from a number of unusual diseases that are rarely reported in other captive felids. Despite intensive efforts by veterinarians and researchers very little progress has been made in the last thirty years in the development of our understanding of the underlying mechanisms of disease in this species. As I considered this problem, it became clear that a new approach was needed. Henrik Kacser, a Romanian-born biochemist once wrote "*But one thing is clear: to understand the whole, one must study the whole*". It was clear to me that we needed a better understanding of cheetah metabolism before we could begin to comprehend the disease processes at play in these animals. Metabolomic studies increasingly provide key baseline information and a means of generating new hypotheses, in what has been termed a "systems biology approach" and this is the approach I took in this study.

Aims and objectives

The aim of the study was to establish a comprehensive serum and urine metabolome for both captive and free-ranging cheetahs and to evaluate metabolite differences in terms of age, sex, body condition and captivity status. The objectives therefore included the sampling and analysis of both serum and urine samples from both captive and free-ranging cheetahs using a combination of gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry platforms. Metabolites evaluated included serum and urine amino acids, urine organic acids, serum fatty acids and serum acylcarnitines.

Structure of the thesis

The introduction provides a background to the various chronic disease problems suffered by cheetahs in captivity. This is followed by a chapter in which I discuss a novel method I used to approximate body condition in cheetahs. In the third chapter, I provide arguments, based on my findings in cheetahs, for the use of urine specific gravity rather than urine creatinine concentrations to correct spot urine metabolite concentrations. In Chapters 4, 5 and 6, I report on and evaluate the amino acid, organic acid, fatty acid and acylcarnitine metabolites found in cheetah serum and/or urine samples. Chapter seven provides an overall summary and discussion of the findings.

Summary of samples collected

A total of 58 urine samples were collected during 2012 and 2013 from captive and free-ranging cheetahs at the AfriCat Foundation in Namibia and from captive cheetahs at the National Zoological Gardens of South Africa. Fifty-seven of these were used for the urine specific gravity, urine creatinine and urine organic acid analyses described in Chapters 3 and 4.

Forty-two serum samples were collected from captive cheetahs housed at the AfriCat Foundation in 2013. Urine samples were simultaneously collected from 26 of these individuals (included in the 58 samples described above). These samples were used for the amino acid analyses described in Chapter 5, while 35 of these were included in fatty acid and acylcarnitine analyses (Chapter 6).

Serum samples were also made available from 44 free-ranging cheetahs trapped on commercial farmland in central and northern Namibia. Unfortunately no urine samples were available from these animals.

Matching serum and urine samples were thus only available for 26 individuals in this study. In order to increase the sample size, unmatched urine or serum samples were still included for some of the analyses.

Outcomes of the study

Two articles have been published from this study so far (see Appendix 1):

> Comparative serum fatty acid profiles of captive and free-ranging cheetahs (*Acinonyx jubatus*) in Namibia, was published in PLoS One in December 2016.
>
> Gas chromatography-mass spectrometry profiles of urinary organic acids in healthy captive cheetahs (*Acinonyx jubatus*), was published in *the Journal of Chromatography B* in February 2017.

A third manuscript entitled Serum and urine amino acid profiles of captive cheetahs (*Acinonyx jubatus*) has been submitted to the Journal of Veterinary Clinical Pathology

Author and study leader contributions

The study was conceived by myself, together with my supervisor (Professor L.J. Mienie) and co-supervisor (Professor F Reyers). I immobilized and collected the data and samples from all the captive cheetahs and some of the free-ranging cheetahs at the AfriCat Foundation in Namibia. Additional serum samples from free-ranging cheetahs in central and northern Namibia were

provided by Dr Bettina Wachter and Dr Sonja Heinrich from the Leibniz Institute for Zoo and Wildlife Research. I assisted technicians at the Potchefstroom Laboratory for Inborn Errors of Metabolism with the sample processing. Professor Mienie checked and aligned the results. He also identified some of the unknown compounds. Mari van Reenen performed the PCA and PLS-DA analyses on the urine organic acid data. I performed all the remaining statistical analyses and wrote the manuscripts for the journal articles as well as the thesis. I received some assistance in the structuring and statistical analyses of the cheetah fatty acid manuscript.

Acknowledgements

This work would not have been possible without the dedication and commitment of the directors and staff of the AfriCat Foundation who have clearly shown their passion for cheetah conservation and welfare. I am forever grateful to the Hanssen family for their kindness and support over the years. In particular, I would like to thank Donna Hanssen, Tammy Hoth, Tristan Boehme, Wayne Hansen, Janek Hoth, Selma Amadhila, Chris Moshosho and many others at Okonjima, who have always made me feel at home and shown their commitment to the project.

This project would also not have materialised without the funding and expertise of my supervisor, Professor Japie Mienie. Thank you for patiently introducing me to the field of metabolomics and opening up a new world of possibilities. Thanks also to Professor Fred Reyers for taking the initiative in this venture and for your valuable mentorship.

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I am indebted to Jano Jacobs, Ansie Mienie and others at the Potchefstroom Laboratory for Inborn Errors of Metabolism for their technical support and for doing the bulk of the laboratory analyses. My appreciation also extends to Mari van Reenen for her assistance with some of the more complex statistics.

I would like to express my appreciation to Bettina Wachter and Sonja Heinrich for providing the valuable free-ranging cheetah samples.

I am eternally grateful to my parents, Eric and Mallory Tordiffe. Thank you for your unwavering love and belief in me. I owe similar gratitude to my in-laws, Bill and Vandra Buckle for their love and support. To my children, Kate, Ross, Georgia and Seth, thank you for hanging in there when I have been absent in mind or presence. I love you all very much.

My deepest gratitude is reserved for my wife and soulmate, Ashleigh. Thank you for the sacrifices you have made and for listening to my late night ramblings with understanding and enthusiasm.

Your love and encouragement has been a deep source of strength and has carried me through on this journey.

Lastly, I would like to thank God for leading me on a path beyond my own dreams and expectations. In the words of the psalmist, “What is man, that thou are mindful of him?” I am overwhelmed by your grace.

SUMMARY

In captivity, cheetahs (*Acinonyx jubatus*) are known to frequently suffer from several chronic diseases including lymphoplasmacytic gastritis, glomerulosclerosis, renal amyloidosis, veno-occlusive disease of the liver, adrenal hyperplasia and several ill-defined neurological disorders that are rare in free-ranging animals. Stress, lack of exercise, low genetic variability and the provision of unnatural diets in captive facilities have been proposed as potential causal factors, but to date convincing pathophysiological explanations for these diseases have been lacking or unsatisfactory. Using a systems biology approach, we used untargeted metabolomic analysis of serum and urine from captive and free-ranging cheetahs, generating new physiological data for this species in the hope of developing a better understanding of their metabolism.

Prior to the actual quantification of serum or urine metabolites, we created a new, more objective, method of approximating body condition in cheetahs by means of a body mass index value. As expected, the morphometric data and body mass indices obtained in the study population showed significant differences between males and females, but importantly, all the animals fell within a healthy body mass index range. The impact of body condition on various serum and urine metabolites could thus be objectively assessed. We also evaluated the use of either urine creatinine concentrations or urine specific gravity values for the correction of spot urine samples obtained from the cheetahs. Creatinine excretion was found to be highly variable in the study animals - influenced by individual, largely age-related differences in creatinine production rather than accurately reflecting changes in glomerular filtration rate. Relying on urine creatinine concentrations to correct or “standardize” urine metabolite concentrations would therefore result in overestimation of metabolite concentrations in younger and older cheetahs. The variability in urine specific gravity was considerably lower and shown to provide a better indication of urine dilution. Urine specific gravity was therefore used to correct urine metabolite concentrations in this study.

Using gas chromatography-mass spectrometry, 339 different organic acids were annotated and quantified in the urine of 56 captive and two free-ranging cheetahs. Phenolic compounds, thought to be produced by the anaerobic fermentation of aromatic amino acids in the distal colon, as well as their corresponding glycine conjugates, were present in high concentrations in the urine of the captive cheetahs. It is suggested that the required detoxification of these phenolic compounds through glycine conjugation could result in the chronic depletion of both glycine and sequestration of Coenzyme A, with associated negative metabolic consequences. We suggest that the high urine levels of these phenolic compounds may be caused by an excess in dietary protein as most captive cheetahs are fed a diet rich in muscle meat and low in fat and other so-called animal fibre.

Concentrations of these phenolic compounds correlated negatively with the end-stage metabolites of dopamine and catecholamines, providing a potential mechanism for significant neuroendocrine dysregulation. Potential mechanisms by which dopamine depletion may play a central role in the pathophysiology of both gastric and renal disease in cheetahs are discussed.

Using gas chromatography-mass spectrometry as well as liquid chromatography-tandem mass spectrometry we established serum and urine amino acid profiles in captive cheetahs. Although the serum concentrations of most of the amino acids in cheetahs were comparable to those in published data for domestic cats, the serum arginine and ornithine concentrations were substantially higher.

Finally, the serum fatty acid and acylcarnitine profiles of 35 captive and 43 free-ranging cheetahs were evaluated through the use of gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry. The profiles obtained from the free-ranging animals provide a unique, healthy control group for comparison. Significant differences were noted for most of the fatty acid and acylcarnitine concentrations between these two populations, indicating dramatic differences in the dietary fat intake, composition and/or metabolism of these nutrients. Most of the serum polyunsaturated fatty acid and mono-unsaturated fatty acid concentrations were significantly lower in the free-ranging cheetahs, compared to the captive animals, suggesting that the fatty acids in the wild cheetah diet are largely saturated. Fatty acids not only provide a valuable source of energy, but also perform other vital functions in the body, including hormone production, cellular signalling and the provision of structural components of biological membranes. Altered serum fatty acids could thus have a dramatic impact on health and, since their concentrations are largely influenced by diet, the values obtained from free-ranging cheetahs potentially provide valuable healthy target values for their captive counterparts.

Through this unique approach, we have established new baseline data for a large range of serum and urine metabolites in cheetahs. The results raise many questions and provide valuable new insights and hypotheses into the potential mechanisms of metabolic disorders in captive cheetahs, creating a platform for future research in this species.

Key terms - cheetahs, *Acinonyx jubatus*, captivity, metabolomics, chronic diseases, body mass index, serum, urine, creatinine, specific gravity, organic acids, amino acids, fatty acids, acylcarnitines.

ETHICAL AND PERMIT CONSIDERATIONS

The project was approved by the National Zoological Gardens of South Africa's Research and Ethics Committee (Project no. P11/07). A research/collecting permit (1846/2013) was obtained from the Namibian Ministry of Environment and Tourism and the samples were imported into South Africa with the required CITES export (no.0042838) and import (no. 137670) permits, as well as a veterinary import permit (no. 13/1/1/30/2/10/6-2013/11/002397). Once in South Africa, the samples were transported and stored with the required national Threatened or Protected Species (TOPS) ordinary permit (no. 05238).

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5-IAA	5-Indoleacetic acid
ACR	Absolute urine creatinine concentration
AGEs	Advanced glycosylation end-products
AD	Alzheimer's disease
ATP	Adenosine triphosphate
BH4	Tetrahydrobiopterin
BL	Body length
BMI	Body mass index
BCFA	Branched-chain fatty acid
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CCR	Corrected urine creatinine concentration
CoA	Coenzyme A
CR	Creatinine
DHA	Docosahexaenoic acid
DHPR	Dihydropteridine reductase
DMT	N,N-dimethyltryptamine
EI	Electron ionisation
EPA	Eicosapentaenoic acid
FA	Fatty acid
%FE	Fractional excretion
FG	French gauge

GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HbA1C	Glycosylated haemoglobin
HPA	Hypothalamus-pituitary-adrenal
HVA	Homovanillic acid
IUCN	International Union for Conservation of Nature
Km	Kilometre
L	Litre
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
NFTs	Neurofibrillary tangles
NMR	Nuclear magnetic resonance
m	metre
MCADD	Medium-chain acyl co-enzyme-A dehydrogenase deficiency
MDD	Major depression disorder
MHC	Major histocompatibility complex
MHPG	3-Methoxy-4-hydroxyphenylglycol
Mmol	Millimole
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
MS-QQQ	Triple quadrupole mass spectrometer
MTBSTFA	N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide
MUFA	Mono-unsaturated fatty acid

PCA	Principal components analysis
PLS-DA	Partial least squares-discriminate analysis
PUFA	Polyunsaturated fatty acid
q-BH2	q-dihydrobiopterin
QC	Quality control
ROS	Reactive oxygen species
SAA	Serum amyloid A
SCFA	Short-chained fatty acid
SFA	Saturated fatty acid
SG	Specific gravity
SH	Shoulder height
TMCS	Trimethylchlorosilane
µmol	micromole
VIP	Variable importance in projection
VMA	Vanillylmandelic acid
VOD	Veno-occlusive disease

CHAPTER 1
GENERAL INTRODUCTION

1.1 Background

Cheetahs (*Acinonyx jubatus*), are highly specialised large felids, known to be the fastest land mammal and the last surviving member of the *Acinonyx* genus. They once ranged across most of Africa, southern Asia and the Middle East, but their numbers have declined sharply over the last few decades and are now only found in about 24% of their original estimated range (1). Cheetahs are currently listed as vulnerable on the International Union for Conservation of Nature (IUCN) red data list. The 2008 IUCN assessment estimated the global population to be less than 10 000 individuals, with the largest populations remaining in southern and eastern Africa. Habitat loss and fragmentation, as well as conflict with livestock farmers are considered to be the primary threats (2). Interspecies conflict with lions, leopards and spotted hyenas also has a dramatic effect on cheetahs with cubs particularly vulnerable to being killed by these more powerful competitors (3).

Female cheetahs are usually solitary and only accompanied by their dependent offspring. The males are either solitary or form small stable coalitions of two to three related or unrelated individuals. Cheetahs generally occur at lower densities (0.2 per 100 km^2 in Namibia farmland (4) and up to 1.0 per 100 km^2 in the Serengeti (5)) than other large carnivores.

Unlike other predators, cheetahs are largely diurnal. Although they are able to hunt prey several times their own body weight, they preferentially prey on abundant small to medium sized antelope (23 to 56 kg in body weight). The smaller prey presumably pose less of an injury risk to cheetahs and can be rapidly consumed before the arrival of other kleptoparasitic carnivores and scavengers (6).

Cheetahs have been tamed and used for hunting and display in many countries across Asia, Europe and Africa for centuries (7). Most, if not all of these animals were captured as adults from the wild. By the end of the 19th century cheetahs had largely disappeared from Asia Minor and large parts of the Middle East. In 1952 the species was declared extinct in India. The earliest record of a cheetah in a formal zoological collection is from The Zoological Society of London in 1829. Between then and 1952, 139 wild-caught cheetahs were displayed at 47 zoological facilities. These animals generally only survived for short periods in captivity and during that 23 year period, 115 deaths and no births were recorded. The first confirmed captive birth of a cheetah

was recorded at Philadelphia Zoo in 1956 (8). Since then only 26% of captive facilities holding cheetahs have reported successful breeding of the species. Between 1956 and 1994, the mortality rate of cheetahs younger than 6 months was high for any zoo bred species at 28%. The average life expectancy had definitely increased by 1994, but was still low with a mean age at death of only 6.1 years (7).

Despite obvious improvements in husbandry since cheetahs were first kept in captivity, they still suffer from a range of unusual diseases not typically seen in other large captive felids. These include glomerulosclerosis (9-12), renal amyloidosis (12), lympho-plasmacytic gastritis (10,13,14), veno-occlusive disease (10,15), splenic myelolipomas, cardiac fibrosis (10,12), adrenal cortical hyperplasia (9,10,12,16) with lymphocytic depletion of the spleen (10), pancreatic atrophy (10) as well as several ill-defined disorders of the neurological system (10,17). Some of these chronic degenerative diseases eventually affect the majority of cheetahs in captivity and are considered to be the primary cause of morbidity and mortality in adult animals (10,18). In contrast, the incidence of similar conditions in free-ranging cheetahs was found to be very low (18).

Stress, lack of exercise, low genetic variability and the provision of unnatural diets in captive facilities have been proposed as potential causal factors, but to date convincing pathophysiological explanations for these diseases have been lacking or unsatisfactory.

1.2 Diseases associated with captivity

1.2.1 Gastritis

An unusual form of gastritis, often associated with *Helicobacter spp*. is a significant cause of morbidity and mortality in captive cheetahs worldwide (10,11). The lesions principally occur in the gastric fundus and are characterised histologically by the infiltration of plasma cells and lymphocytes into the lamina propria and submucosa. In chronic cases, gland hyperplasia, goblet cell metaplasia, fibrosis and/or atrophy may be seen (10,11,14). This lymphoplasmacytic gastritis is unlike the mild *Helicobacter* associated gastritis seen in other domestic and captive wild felids (19,20). Although four different *Helicobacter spp.* have been isolated from cheetahs with gastritis (21), these bacteria are unlikely to be the primary cause of the disease since most free-ranging cheetahs have *Helicobacter*, but do not suffer from significant gastritis (18). In captive cheetahs, no single strain of Helicobacter was associated with gastritis and similar strains were found in cheetahs with and without gastritis (21).

In severe cases, symptoms include chronic vomition, weight loss, diarrhoea and poor coat condition. Vomiting may result in asphyxiation or pneumonia from aspirated food, while the chronic inflammation has been linked to the development of systemic amyloidosis often resulting in renal failure (10-12). Surveys of post-mortem findings in cheetahs have recorded incidences of 91% (1989 – 1992) and 99% (1988 – 2002) in the North American cheetah population (11,18) and 100% (1975 -1995) and 99% (1988 -2002) in South African cheetahs (10,18). In the 1975 to 1995 South African survey, 37% of the deaths were directly attributable to gastritis (10). In contrast, the incidence and severity of the disease at some institutions in South Africa was found to be much lower (22). Temporary improvement in the degree and distribution of gastritis has been achieved in most cheetahs with a three-week course of omeprazole, amoxicillin and metronidazole (23). Long-term follow-up examinations of cheetahs treated for gastritis, however, clearly showed that the treatments had little effect on the progression of the disease and it therefore seems very unlikely that *Helicobacter* is the causal agent of this disease (24).

Aberrant host immune responses due to a lack of helminth enteric infections, low major histocompatibility complex gene diversity, chronic stress and potential dietary causes have been suggested for this disease in cheetahs (21), yet in the 24 years since first reported in cheetahs, an underlying cause for the disease has not been identified.

1.2.2 Glomerulosclerosis

Renal failure is a major cause of death in captive cheetahs (11,25), with glomerulosclerosis as the most common renal lesion detected at post-mortem (10,11,18). Glomerulosclerosis has been found in 67 to 84% of captive cheetahs compared to only 13% of free-ranging cheetahs (9,10,18). Histologically the lesion is characterised by a thickening of the glomerular and tubular basement membranes, eventually deteriorating into glomerulosclerosis. The severity of the lesions increase with age and resemble diabetic nephropathy in humans or chronic progressive nephropathy in rats with accumulation of advanced glycosylation end-products (AGEs) in the renal basement membranes of cheetahs (9). However, diabetes mellitus is rarely diagnosed in cheetahs and has not been reported in the literature. Bolton and Munson suggested that the AGEs may form as a consequence of stress induced hyperglycaemia or the high protein diet and daily feeding of captive cheetahs, but evidence for hyperglycaemia in the form of elevated serum fructosamine or HbA1C concentrations have not been reported in the literature. The pathogenesis of glomerulosclerosis in cheetahs therefore remains unclear.

1.2.3 Amyloidosis

In many species, systemic AA amyloidosis is a rare complication of chronic inflammatory conditions. In amyloidosis, the liver produces a serum reactant acute phase protein called amyloid A (SAA). The precise function of SAA is not known, but it is thought to have modulating effects on reverse cholesterol transport and lipid functions at inflammatory foci. Under normal physiological conditions, SAA is rapidly taken up by macrophages and degraded in the lysosomal compartment. In patients with amyloidosis, this degradation does not take place and SAA intermediates aggregate into fibrils which are deposited into the intracellular spaces of various organs including the liver, pancreas and kidneys (26).

In cheetahs, systemic amyloidosis is often associated with lymphoplasmacytic gastritis, enteritis and colitis (12). The most common site of amyloid deposition in the cheetah is the kidney. The renal amyloid distribution pattern, primarily in the medullary interstitium, is often associated with interstitial fibrosis and tubular atrophy, frequently resulting in chronic renal failure (12). The incidence of this disease in captive cheetahs is high, ranging from 35% to 38% of cheetahs in post-mortem pathology surveys (12,18). Papendick *et al*, noted an increase in the number of cheetahs with systemic amyloidosis presented for necropsy, with an incidence of 20% prior to 1990 compared to 70% in 1995. It is suggested that this parallels the increase in the incidence of gastritis over that period (12). Not all cheetahs with chronic inflammatory conditions, such as gastritis, end up developing amyloidosis. Other factors involved in the pathophysiology of this disease have therefore been suggested including the possibility of it being transmissible, similar to prion disease (27,28). The epidemiology of this disease in cheetahs is however quite complex and simple transmission seems unlikely (29).

1.2.4 Veno-occlusive disease

Unusual hepatic diseases in captive cheetahs have been reported in the literature since the late 1960's (15). Post-mortem liver tissues from 126 captive adult cheetahs, collected between 1945 and 1986, mostly from North American zoos, showed a 60% incidence of veno-occlusive disease (VOD). Vascular lesions of the hepatic central veins, characterised by the proliferation of smooth muscle-like cells and the accumulation of fine fibres, leading eventually to partial or complete occlusion of the veins were described (15). In South African captive cheetahs, the incidence of VOD was slightly lower, ranging from 36% to 43% of the population (10,18).

The most common cause of VOD in other species is pyrrolizidine alkaloid toxicity, yet megalocytosis a common feature of pyrrolizidine alkaloid toxicity was not found in any of the

cheetah VOD livers (15). Chronic hypervitaminosis A has been suggested as a possible cause for VOD in cheetahs since vitamin A excess in humans is known to cause perisinusoidal fibrosis and a proliferation of the liver Ito cells that are known to store vitamin A. Gosselin *et al* recorded in 7 out of 9 cheetahs, hepatic vitamin A concentrations 5 to 19 times higher than the upper limits for domestic cats (15). However they noted discrepancies in the increase in Ito cells and the severity of VOD in cheetahs in the study. In cheetahs with VOD, 30% showed no evidence of Ito cell proliferation. The authors concluded that although hypervitaminosis A may play some role in VOD in cheetahs, other as yet unidentified factors are likely to be involved in the pathogenesis.

1.2.5 Neurological diseases

A number of central nervous system disorders have been identified in captive cheetahs. These include encephalomyelopathy and leukoencephalopathy (17). In a recent study, brain lesions similar to those seen in human Alzheimer's disease, were described in this species (30).

Encephalomyelopathy has emerged primarily in European facilities in the last 25 years. It is characterised by symmetrical degenerative lesions in the spinal cord and cerebellum with loss of myelin, resulting in ataxia and paresis. The disease was responsible at one stage for 25% of the deaths in cheetahs within the European captive breeding programme. The disease affects cheetahs of all age groups, often siblings are affected simultaneously or successively over a period of months to years. The etiology of this disease is currently unknown. Genetic, environmental, toxic, viral and nutritional factors have been considered, but no common denominator has yet been identified (17).

Leukoencephalopathy appeared in the North American captive cheetah population in 1994, affecting 73 mature adults over a 9-year period. Since then, no new cases have been recorded. The primary clinical symptoms were progressive loss of vision, disorientation and difficulty eating. The symptoms became more severe at variable rates over days to years. Bilateral degenerative lesions were seen on histopathology in the cerebral white matter and to a lesser extent in the white matter of the brainstem and spinal cord. No underlying cause was ever established, but ingestion of an unknown neurotoxin was suggested as the most likely cause (31).

The three typical histologic features of Alzheimer's disease (AD) in humans; cerebral atrophy with neuronal loss, senile plaques consisting of Aβ deposits and neurofibrillary tangles (NFTs), have recently been described in the brains of 13 aged captive cheetahs in Japan (30). Individual features of AD, such as the senile plaques have been recorded in aged domestic felids and other animals before, and two of the three features have been described in a few individual case

reports, but other than in humans, cheetah appears to be the only animal in which all three features spontaneously develop. Interestingly only two of the 13 cheetahs in the study showed subjective evidence of cognitive dysfunction prior to death. As yet no cause for these neurodegenerative lesions has been found.

1.2.6 Adrenocortical hyperplasia

Adrenocortical hyperplasia is a common finding in cheetahs at necropsy (9-11), but may be associated with the physiological stress suffered due to a number of chronic disease conditions in this species. Terio *et al* evaluated the adrenal glands of 13 captive North American cheetahs that died acutely, without any history of chronic disease. These were compared to the adrenals of 13 free-ranging cheetahs on Namibian farmland. The corticomedullary ratios of the captive animals were significantly larger than the free-ranging cheetahs. The findings were supported in the same study by higher faecal corticosteroid concentrations in captive cheetahs compared to the free-ranging Namibian cheetahs (16). In other studies however, no significant difference was found between the overall sizes of the adrenals in captive Namibian cheetahs and their free-ranging counterparts (32). Several adrenal measurements (cranial pole and cortical widths) have recently been shown on transabdominal ultrasound to increase relative to age in Namibian captive cheetahs (33). Although various environmental and social stressors may lead to adrenal enlargement, other metabolic and age-related factors may also play a role in adrenocortical hyperplasia in this species.

1.2.7 Other abnormalities

Other common findings with unknown etiologies in captive cheetahs include lymphocytic depletion of the spleen, myelolipomas of the liver and spleen as well as cardiac fibrosis.

Hepatic and splenic myelolipomas are only rarely reported in domestic species (34,35). Histologically, the splenic or hepatic masses in cheetahs are better described as nodular lipomatosis, since only a relative increase in megakaryocytes was found in tissue adjacent to the lesions (36). The incidence of these lesions in cheetahs varies from 14% in South African cheetahs (10) to 48% in North American cheetahs (11). It has been suggested that endocrine abnormalities, such has hyperadrenocorticism and/or chronic disease are required for myelolipomas to form in humans (37). Clear associations with specific diseases in cheetahs however remain unclear.

Both lymphocytic depletion of the spleen and cardiac fibrosis are suggested to be the result of chronic stress in cheetahs (10), though these conditions in cheetahs have not been specifically investigated in any study to date.

1.3 Genetic diversity

Since the 1980's, much was made of the apparent low genetic diversity of the cheetah and it has often been reported widely as an example of increased disease vulnerability due to inbreeding depression (38,39). This genetic uniformity has been attributed to the species having gone through a demographic crisis or population bottleneck near the end of the last ice age (40). It has been suggested that cheetahs are more vulnerable to infectious diseases due to low variation in their major histocompatibility complex (MHC) genes which contribute to adaptive immune system function (41). Outbreaks of feline infectious peritonitis in the North American captive population between 1982 and 1983, as well as severe clinical symptoms and high mortality due to feline panleukopaenia, feline herpesvirus and canine parvovirus, seemed to support this theory (42-44). However, captive and free-ranging cheetahs share the same recent ancestry, with comparable genetic variation across populations (39,45) and yet despite widespread exposure to infectious agents (46), wild cheetahs were found to be remarkably free of disease (18). Furthermore, to date, no heritability has been demonstrated for any of the non-infectious disease associated with captivity. A primary genetic basis for the high incidence of disease in captive cheetahs therefore seems unlikely.

The low fecundity, poor breeding record and high infant mortality rates have also been put forward as evidence for the genetic impoverishment of captive cheetahs (38). Juvenile mortality rates in captive cheetahs were however shown to be lower than six other captive felid species. In the same study captive cheetahs were shown to produce larger litters than other felids, with higher average numbers of cubs surviving per litter. Inbred cubs, from related parents, suffered significantly higher mortality rates than non-inbred offspring. This is in contrast to predictions that the low genetic diversity of cheetahs would result in little or no difference in survival rates in cubs from related versus unrelated parents. Inbred cubs were also more likely to die of intrinsic factors such as stillbirths and congenital defects than non-inbred individuals. The authors argue convincingly that captive cheetahs still have sufficient variation at the genetic loci responsible for juvenile survival to cause significant differences in cub survival rates between inbred and non-inbred individuals (47). Although the juvenile mortality rate of wild cheetahs is high, with only 4.8% of cubs reaching maturity in East Africa, this has largely been attributed to predation by lions and

hyenas (28). In Namibia, where the density of other large predators is low, 75% of cheetah cubs survive to the age of 12 months (29).

The reproductive rates of captive cheetahs have increased in the last few decades, but are still considered to be low. Prior to 1993, only a third of female cheetahs in North America ever produced offspring and half showed minimal or no ovarian activity (48). Since then, only a few institutions have managed to consistently produce cubs (25,49,50). In contrast, free-ranging cheetahs reproduce readily and have high cub survival rates, especially in the absence of other predators (5,32,51). Both captive and free-ranging cheetahs produce similarly large proportions of defective spermatozoa and have comparable sperm motility and concentration characteristics (52). This does not however seem to hamper conception since a high proportion of cheetahs (17 out of 19 females) have been shown to conceive after a single oestrus (53). These findings suggest that extrinsic factors such as husbandry, breeding management, potential stressors or nutrition factors are likely to be responsible for the poor reproductive performance of cheetahs in captivity rather than factors intrinsic to the species in general.

1.4 Stress

Many have argued that captivity is simply too stressful for the cheetah and that physical, social or psychological stressors are at least partially to blame for the poor reproductive performance and health of this species in captivity. This theory was supported by a study that demonstrated large differences in faecal glucocorticoid concentrations and post mortem adrenal morphometrics between free ranging Namibian cheetahs and cheetahs in North American zoological facilities. The baseline mean corticoid concentrations were significantly higher and adrenal corticomedullary ratios larger in the captive cheetahs. The authors argued that these findings provided both functional and morphological evidence that captive cheetahs are under chronic stress (16). Adrenocortical hyperplasia is a common post mortem finding in captive cheetahs in South Africa and North America with an incidence of between 56 and 83% (9,10,18).

The accommodation of cheetahs in zoo display enclosures seems to have some influence on stress levels. Cheetahs moved to on-exhibit enclosures had elevated faecal glucocorticoid levels compared to cheetahs moved to off-exhibit enclosures in the 30 day period post translocation (54). In another study, cheetahs housed in off-exhibit enclosures produced more motile sperm per ejaculate than cheetahs in enclosures exposed to the public (55). Surprisingly, in that study however, the faecal glucocorticoids did not differ between the on-exhibit and off-exhibit groups. Captive cheetahs accommodated in large bushveld camps in Namibia, with little public exposure, were shown to have adrenal glands of similar size to their free-ranging counterparts (32). Faecal

corticosteroid concentrations in a similar group of captive Namibian cheetahs were also shown to be lower than those typically recorded from captive cheetahs in North American zoos (56)

Other potential stress factors, such as the abnormal grouping of particularly female cheetahs and the close proximity of other large predators in adjacent enclosures have also been proposed (57,58). Although the pairing of female cheetahs has been shown to suppress ovarian cyclicity and cause behavioural changes, it had little effect on faecal glucocorticoid excretion (58). The excretion of faecal corticosteroids and behavioural changes thought to be associated with stress appear to be highly variable and somewhat dependent on individual temperament (54). The stress response in cheetahs also seems to involve more than a simple activation of the hypothalamus-pituitary-adrenal access, affecting reproductive aspects, but not corticosteroid production. Clearly, some cheetahs in captivity are stressed by environmental, social or husbandry factors, but it seems unlikely that physiological stress alone can explain the high levels of chronic disease in these animals. The level at which cheetahs are exposed to the public, differ between the zoo like conditions in North America where some animals appear to have enlarged adrenal glands and high faecal cortisol levels (16) and the Namibian captive cheetah populations where both adrenal measurements and faecal cortisol levels are comparable to free-ranging cheetahs (32,56). The incidence of chronic disease is however equally high in both populations. Stress and/or chronic elevation of cortisol levels therefore do not appear to be the primary cause for the diseases suffered by captive cheetahs.

1.5 Nutrition

Like other felids, cheetahs are hypercarnivores. In the wild they preferentially prey on small to medium sized antelope, ranging from 23 – 56kg, with a mean mass of 27kg (6). If left undisturbed, cheetahs will consume most parts of the carcass, including the skin, internal organs, muscle meat and most of the bones, leaving only the skull and gastro-intestinal tract of larger prey (59). After opening the abdomen they generally remove the gastro-intestinal tract and then preferentially feed on the abdominal organs and fat before consuming the muscle meat of the back, hindquarters, forelimbs and neck (59-61). African antelope species store most of their fat reserves inside the abdomen and it may well be that cheetahs target this energy rich resource together with the nutrient rich abdominal organs to maximize their nutrient intake before losing their kill to other predators. Selective consumption of body parts has been noted in several other species and may reflect the need of predators to regulate their macronutrient intake (62).

In captivity, cheetahs are either fed commercially prepared diets that are mostly formulated according to the requirements of the domestic cats or various supplemented raw meat diets. In

one study, whole carcasses only made up 21% of the diets fed to cheetahs in European zoos, 5% of the diet in African facilities and were absent from the cheetah diets in North American facilities (63). Commercial carnivore diets are most popular in North America where they make up the largest proportion of the food fed to cheetahs (63). In South Africa and Namibia, captive cheetahs are mainly fed the eviscerated and exsanguinated carcasses of several domestically farmed species. This carcass meat is generally supplemented with a variety of powdered minerals and vitamins. At some institutions commercial pelleted cat foods are provided, but these diets are expensive, if not donated by the manufacturers, and known to cause diarrhoea if they make up a large proportion of the total diet (64).

Vitamin and mineral deficiencies are often suspected in captive cheetahs, but rarely confirmed in practice due a lack of suitable laboratory facilities, the extensive time required before results are available, and a lack of reliable reference ranges and the cost of these analyses. Suspected or confirmed cases of mineral or vitamin deficiencies in cheetahs include metabolic bone disease due to calcium deficiencies and/or hypovitaminosis D (65), deficiencies in vitamin A (66), copper (67) and taurine (68). Ulnar metaphyseal osteochondrosis in young captive bred cheetahs was thought to have occurred due to over-supplementation with calcium (69).

Unlike the domestic cat, the nutritional requirements of the cheetah have not been determined. Feral cats that hunt small mammals and birds would typically consume several small prey every day (70). Cheetahs on the other hand, consume more sizeable prey relative to their own body weight and rarely eat every day (60). The differences in feeding frequency between these felid species is likely to result in several metabolic differences, but given the dearth of nutritional and metabolic information available for the cheetah, the domestic cat certainly provides the most accurate available model.

Domestic felids have been shown to have high requirements for certain key nutrients that are considered non-essential for other less carnivorous mammals (71,72). Cats are metabolically adapted to preferentially get their energy requirements from animal derived proteins and fats. Carbohydrates consumption would normally be low (1-2%) in the form of glycogen stored in the liver and muscles of their prey (73). Adult cats require 2 to 3 times more protein in their diet than dogs, largely due to a higher basal demand for nitrogen and an increased requirement for several essential amino acids (73). On a low protein diet, omnivores conserve their protein stores by reducing the activity of the enzymes involved in protein catabolism. Although cats are able to adapt to different levels of protein intake, they continue to utilize protein stores for energy even when fed very low protein diets (74,75). Cats require larger amounts of specific amino acids such as arginine, taurine, cysteine and methionine in their diet (73). As these amino acids are typically found in sufficient supply in most prey species, metabolic pathways for their synthesis would be

largely redundant. Cat also utilize animal fats very efficiently for energy, but require key essential fatty acids such as linoleic, linolenic and arachidonic acid, as well as eicosapentaenoic acid which they are unable to synthesise sufficiently themselves (76). Cats have higher dietary requirements for thiamine, niacin and pyridoxine than dogs (77). Both vitamin A (retinol) and vitamin D_3 (cholecalciferol) are normally only found in certain animal tissues such as the liver, fat and skin. Unlike omnivores and herbivores, cats do not have the required enzymes to convert the precursors (β-carotene and ergocalciferol), typically found in plants, into the required biologically active vitamins (73).

Given these key nutrient requirements, felids are believed to be metabolically inflexible and their inability to synthesize several key nutrients place them at an increased risk of nutritional or metabolic disease. Acute nutritional deficiencies often results in dramatic clinical symptoms and although they may be challenging to diagnose, once treatment is initiated, patients often respond rapidly to supplementation and rarely suffer significant long-term effects (78). Chronic diseases related to suboptimal nutrition, on the other hand, often have more complex etiologies and are far more difficult to treat. In humans, for example, the role of nutrition in the pathophysiology of heart disease, diabetes, strokes, dementia and various forms of cancer have been the subject of much debate and research and yet there is little consensus on nutritional recommendations which would prevent these diseases.

In terms of captive cheetahs, the research focus has shifted in recent years to the exploration of the epidemiological links between diet and disease (63,79). Such research is hampered by small sample sizes, great variation in the dietary components that are fed to cheetahs, difficulty in obtaining definitive diagnoses of both gastritis and early renal disease, as well as the slow progression of these diseases. The provision of whole carcasses has recently been shown to improve faecal consistency in captive cheetahs compared to when a meat-based diet or commercially prepared diets were fed (63,80). Cheetahs fed whole rabbit carcasses produced higher faecal short chain fatty acid (proprionic acid and butyric acid) concentrations and lower putrefactive products (indole and phenol) than those fed supplemented beef (80). The differences in this study were ascribed to the positive effects and fibre-like functions of the additional hair, skin, bones and cartilage provided by the whole rabbit diet. Short chain fatty acids like butyric acid, are known to provide an important energy source for colonocytes, stimulating blood flow and motility and decreasing the growth of pathogenic bacteria (81,82). Despite the apparent advantages of feeding whole carcasses, this food type is more expensive to obtain and keep fresh prior to feeding.

These epidemiological studies seem to indicate a link between the macronutrient components in cheetah diets and gastro-intestinal health. However, direct studies evaluating the link between

nutrition and gastritis, renal disease or other chronic abnormalities in captive cheetahs are still lacking and very little is known about the potential pathophysiological mechanism involved.

1.6 Exercise

There is little doubt that free-ranging cheetahs utilize more energy searching for, chasing and killing prey than their captive counterparts. This lack of exercise has been proposed as a potential contributing factor in the diseases of captive cheetahs (18). Lures have been used as a form of behavioural enrichment in so-called “cheetah runs” to encourage normal chasing behaviour and increase levels of fitness (83). However, no study has evaluated the direct health benefits or the impact of such practices on the incidence of any of the chronic diseases suffered by captive cheetahs.

1.7 Zoological non-infectious disease epidemiology

Classic disease investigations in zoological medicine rely on the fields of epidemiology and comparative medicine and are largely hypothesis driven. In the case of infectious diseases, causal organisms are often visualised directly in affected tissues or indirectly through serological or molecular techniques. These infectious agents can sometimes be frustratingly difficult to control, as in the case of *Mycobacterium tuberculosis* (84), but rarely is the determination of causality a problem. For non-infectious diseases the epidemiological challenges are however substantial. In typical zoological disease investigations, pathological lesions are characterised and literature on similar disease patterns in better studied species sought in order to generate potential hypotheses (9,85,86). If feasible and ethical, these hypotheses are then tested. Occasionally disease etiologies are identified, but very often the results are ambiguous and clinicians and researchers are left frustrated, without clear answers. Some new knowledge may be obtained through the process and of course alternative hypotheses can also be investigated, but in most cases, progress is painfully slow in elucidating the underlying causes of these diseases.

Non-infectious disease investigations in zoological species are hampered by several factors; 1) The population sizes are often very small and housed at multiple institutions, each with their own husbandry and management practices and sufficient animals are rarely available for properly controlled clinical trials. Epidemiological studies therefore require multi-institutional cooperation and data are often collected subjectively by numerous people (63). The potential number of confounding variables under these conditions, further reduces the statistical power of the findings. 2) Many of the animals are not tame enough to allow routine sample collection (blood, urine etc.)

without immobilisation or sedation. Not only does immobilization cause additional stress to the animals, but the administration of anaesthetic drugs may influence several sample parameters, masking changes associated with the disease being investigated. 3) These valuable animals cannot be sacrificed for post-mortem evaluation and invasive sampling techniques are generally not approved by animal ethics committees. 4) The diseases under investigation are often chronic and require long-term, expensive studies.

In the case of the cheetah, these factors are further complicated by the uniqueness of this species. From the discussions above on the various diseased suffered by this species in captivity, it is apparent that comparable disease syndromes are rarely seen in any other species. The cheetah's separate classification in the genus *Acinonyx*, is apparently not only supported by its distinctive morphology and genetics, but also by the diseases they suffer in captivity. The survival and longevity of cheetahs in captivity has without question improved over the last 30 years, but little progress has been made in understanding the causes and pathophysiology of the diseases they suffer. The slow progress has not been due to a lack of research. A search on Scopus, using the search term "cheetah" and limiting the results to the "veterinary" field, yielded 197 results between 1972 and 2015, with an average of more than 11 publications per year since the year 2000. Cleary different approaches to this problem are warranted.

1.8 Systems biology approach using metabolomics

"*But one thing is clear: to understand the whole, one must study the whole*" (H. Kacser) (87)

Scientific advancement is dependent on a continuous cycle of linked ideas (hypotheses) and observations (data). The hypothetico-deductive mode of reasoning uses baseline data to generate a hypothesis that can then be tested experimentally to generate observations. This system is however dependent on having sufficient baseline data in order to generate reasonable hypotheses.

The reductionist view in science prefers to break the system up into its component parts, attempting then to reconstruct the system intellectually. This is commonly referred to as the "bottom-up approach". The systems biology, holistic approach or "top down" approach, studies the complex intact system as a whole and hopes to generate hypotheses as a result of the epidemiological study of interest. In this system, hypotheses are thus the goal and not the starting point. Both systems are of course complimentary (88).

The diseases of captive cheetahs have left us with few clear hypotheses to explore and it is clear that we need to broaden our knowledge of their basic physiology in order to create a sound

platform for the generation of new ideas. The field of metabolomics provides a potential means by which such baseline data can be generated. Metabolomics involves the unbiased quantitative and qualitative analysis of the complete (or near-complete) set of metabolites (products of cellular metabolism) present in a cell, body fluids or tissues (89). Such metabolites may include amino acids, organic acids, fatty acids, sugars, carnitines and nucleotides. Together they are referred to as the metabolome. Metabolites may range tremendously in concentration, mass and polarity (90) and a range of technologies including nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS) and Liquid chromatography-mass spectroscopy (LC-MS) are used. These analyses generate vast amounts of data consisting of a range of metabolites (variables) for a number of individuals (observations). Detecting changes in metabolite concentrations across such large and complex datasets is not without its challenges. A variety of multivariate statistical techniques are used in metabolomics. Unsupervised techniques, such as principal components analysis (PCA), model intrinsic variation within a dataset without take class membership into account. Supervised techniques, such as partial least squares-discriminate analysis (PLS-DA), use prior knowledge of classes or groups to maximize separation. These multivariate analyses are often combined with more targeted univariate analysis to provide a better understanding of the results.

Examples of veterinary-based studies in metabolomics are currently still rare in the literature (90). Out of 15 600 results in a keyword search on Scopus for the term “metabolomics”, only 50 remained after the results were limited to the “veterinary” field. Metabolite fingerprinting of urine in domestic dogs has been used to demonstrate metabolic and nutritional differences between breeds (91), and the urine metabolite changes in aging and calorie-restricted dogs have also been studied using nuclear magnetic spectroscopy (92). Metabolomics studies in dogs have evaluated metabolite changes in acute diarrhoea (93), inflammatory bowel disease (94) and degenerative mitral valve disease (95). Serum metabolites have been evaluated in dogs and cats before and after dietary glucose supplementation (96) and in another nutritional study in cats, distinct plasma metabolite profiles were demonstrated in response to differences in dietary macro-nutrient content (97).

To the author’s knowledge, comprehensive metabolic profiles of zoo or other captive wildlife species have not been reported in the literature and only a handful of limited metabolomics studies have been conducted on free-ranging species (98-100). Research on the urine metabolites in wild felids have been limited to the study of compounds that are considered important in territorial marking and other behaviours (101). In an essay in Zoo Biology entitled, “Metabolomics has great potential for clinical and nutritional research with exotic animals”, Dove recognizes the potential for metabolomics studies in this setting (102).

In this study, we analysed both the serum and urine from captive and free-ranging cheetahs in Namibia and South Africa using un-targeted GC-MS and MS-MS metabolomics. Metabolites evaluated in the urine included organic acids and amino acids, while serum was evaluated for amino acids, long chain fatty acids, very long-chain fatty acids and carnitines. Variables were evaluated relative to changes in age, body condition, sex and captive status. The findings provide a baseline of reference values in cheetahs and provide several new hypotheses for consideration in future studies.

CHAPTER 2
MORPHOMETRICS AND BODY MASS INDICES

2.1 Introduction

Serum and urine metabolites are potentially influenced by intrinsic factors such as age, sex and body condition in healthy individuals (103-105). The possible influence of these variables has to be determined before considering the impact of prospective pathophysiological processes. In human studies, a third of 507 blood metabolites differed significantly between males and females (106). The impact of age on the human metabolome has also been studied in infants (107,108) and in aged individuals (109), while body composition has been shown to influence the metabolome both in cases of excess body fat (110) as well as in loss of muscle mass (sarcopenia) (111).

Sex determination in cheetahs is obviously straightforward and although the age of individual free-ranging cheetahs may be difficult to ascertain, accurate ages are generally available for cheetahs in captivity. In contrast, body condition, in terms of muscle mass and body fat percentage, is far more difficult to objectively determine without the use of sophisticated equipment, such as computer tomography or dual-energy x-ray absorptiometry (112), which are not readily available under field conditions. In domestic cats and dogs, various body condition scoring systems are commonly used in both a clinical and research settings (113,114). Such systems may be well defined, but are still subjective, suffering from significant inter-observer variation (115). Body condition scoring systems, adapted from those used in domestic species, have been developed for large captive felids (116,117). Scores are assigned to individuals on a nine-point scale based on certain criteria. These systems have not been formally validated and potentially suffer from the same problem of inter-observer variation. Body mass to body length ratios in wild cheetahs were compared to a basic condition scoring (excellent, fair and poor) based on estimated body fat, parasite loads and coat condition. For these ratios only a single measurement was used (body length) and although the ratios differed significantly between those in excellent condition versus those in fair or poor condition, the method could not differentiate between the fair and poor condition categories (118).

The body mass index (BMI) was first used in human studies in the 1800s (119), but gained popularity for use as the best proxy, at the time, for body fat percentage from 1972 (120). In humans the BMI is defined as the body mass in kilograms divided by the square of body height in meters (kg/m^2). Many have criticised the inherent inaccuracies of this index as a measure of obesity and yet it has been used in numerous research studies. The index relies on a single body measurement in one dimension (height). This measurement is squared in the calculation resulting

in a problem with scalability, in that taller people have relatively higher BMIs, even if they have similar body fat percentages to shorter individuals. Athletes or military personnel with larger muscle mass relative to body height may also be incorrectly classified as overweight or even obese as the BMI does not differentiate between lean muscle mass and total body fat (121).

In order to develop a more objective way of assessing body condition in cheetahs, I developed a body mass indexing system for cheetahs based on body measurements in two dimensions (shoulder height and body length). Neither measurements are influenced by either lean muscle mass or body fat percentage and thus provide a good indication of body size, independent of changes in body condition. When combined with body weight, the calculated value should provide a good approximation of body condition, especially in an animal like the cheetah where body conformation is relatively uniform. In captive cheetahs, relative muscle mass is expected to differ between males and females, but variation within the sexes would be expected to be minimal as the levels of exercise are similar for captive individuals. Thus, changes in BMI are expected to largely reflect variation in total body fat. The method is similar to a BMI system for domestic dogs described by Mawby *et al* (122) and is described in more detail in the materials and methods section below. This method allows for a more objective assessment of the influence of body condition on the metabolome of cheetahs.

2.2 Materials and Methods

2.2.1 Animals

Body measurements were collected from apparently healthy adult cheetahs housed at the AfriCat Carnivore Care Centre near Otjiwarongo in Namibia during routine annual health examinations. All of the cheetahs at the centre were wild-born and rescued as cubs (< 12 months of age) from commercial farmland in central Namibia and therefore were of known age at the time of examination. They either form part of AfriCat's Rescue and Release programme or are deemed unsuitable for release and are maintained in captivity for conservation education and research purposes. Due to Namibian legislation, all the cheetahs had been treated with deslorelin contraceptive implants (Supralorin®, Virbac Animal Health, Australia), a GnRH agonist that downregulates all reproductive hormones in both males and females. The cheetahs are housed in large (> 1000 m^2) camps, covered by natural vegetation and are fed a diet consisting largely of donkey muscle meat supplemented with a combination of vitamins and minerals (FeliCal® and FeliVit®, Kyron Laboratories, South Africa).

2.2.2 Immobilization

The cheetahs were fasted for 12 to 24 hours and immobilized via remote injection dart (Daninject, Denmark) with medetomidine hydrochloride (Medetomidine 20mg/ml, Kyron Laboratories Pty LTD, South Africa) in combination with either zolazepam/teletamine (Zoletil®, Virbac, South Africa) or midazolam (Midazolam 50mg/ml, Kyron Laboratories Pty LTD, South Africa) and butorphanol (Butorphanol 50mg/ml, Kyron Laboratories Pty LTD, South Africa).

2.2.3 Body weights and measurements

All the cheetahs were weighed shortly after immobilization on a walk-on scale (Model: MI301C, Masskot, South Africa). Body length (BL) and shoulder height (SH) measurements were obtained with the animal in lateral recumbency using a 1.5 m flexible plastic measuring tape. The BL measurement was taken from the occiput at the caudal edge of the skull to the base of the tail (at the fold created between the tail and the body when the tail is lifted to 90 degrees), ensuring that the measuring tape remained in contact with body along its entire length. The SH measurements were taken from the dorsal rim of the scapula to the middle of the metacarpal pad with the shoulder, elbow and carpal joints fully extended. The shoulder height was measured to the middle of the metacarpal pad rather than to the point of the toes to allow for more accurate future comparison of measured shoulder heights in anaesthetised cheetahs to those of non-anaesthetised cheetahs in a normal standing position on photographs using photogrammetry.

Using these data a body mass index (BMI) was calculated using the following equations:

$$\textit{Size Index (SI)} = \textit{Shoulder height (m)} \times \textit{Body length (m)}$$

and $$\textit{Body Mass Index (BMI)} = \textit{Body weight (BW) in kg} / \textit{SI}$$

2.2.4 Statistical analysis

Data was tested for normality using the D'Agostino and Pearson omnibus (K2) normality test. Pearson correlation coefficients were used to evaluate the relationship between age and BMI, as well as between body weight and size. Unpaired t-tests were used to compare the ages, weights and morphometric measurements of males and females. All analyses were performed with GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, California, USA). All statistical tests were 2-tailed and significance was defined as $P<0.05$ in all cases.

2.3 Results

Morphometric data were collected from 46 adult cheetahs (26 males and 20 females), ranging in age from 2 to 12 years. A summary of the data is shown in Table 2-1. The mean ages of males and females were similar (6.5 and 7 years respectively) and did not differ significantly (p = 0.59). Male cheetahs were significantly larger (p = 0.0002) and heavier (p < 0.0001) than females. Both shoulder height (p = 0003) and body length (p = 0.0165) were significantly larger in males than in females (shown in Figure 2-1), although in both measurements there was a great deal of overlap between the sexes. Males were also heavier relative to their size than females as demonstrated by their significantly higher BMI values (p < 0.0001) as shown in Figure 2-2.

The correlation between age and BMI was not significant in either males (r = -0.04, p = 0.84) or females (r = -0.23, p = 0.33). The scatter plots showing the correlations between age and BMI in males and females are shown in Figures 2-3 and 2-4.

Table 2-1. Comparative biometric data for the study group of male and female cheetahs

	Males (n=26)				Females (n=20)				
	Max	**Min**	**SD**	**Mean**	**Max**	**Min**	**SD**	**Mean**	**p-value**
Age (years)	12	3	2.9	6.5	13	2	3.5	7.0	0.59
Body weight (kg)	48.5	29.9	4.6	40.25	40.0	29.6	2.64	33.56	< 0,0001*
Size Index (m^2)	0.836	0.605	0.06	0.73	0.765	0.614	0.03	0.67	0.0002*
Shoulder height (m)	0.79	0.68	0.03	0.73	0.75	0.64	0.02	0.70	0.0003*
Body length (m)	1.10	0.85	0.05	0.99	1.0	0.9	0.03	0.96	0.0165*
BMI	60.4	47.16	4.04	55.35	56.16	45.09	3.39	50.22	< 0.0001*

* Statistically significant differences

Figure 2-1. Box and whisker graphs comparing the shoulder heights and body lengths of male and female cheetahs. Whisker represent maximum and minimum values, while boxes indicate median, 25th and 75th percentiles. Mean values are indicated by +.

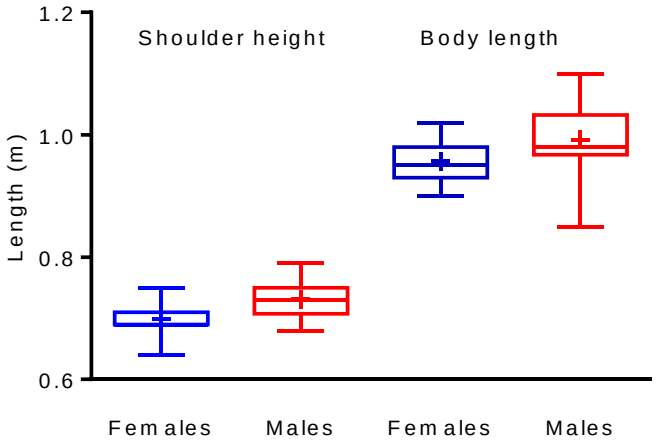


Figure 2-2. Box and whisker graphs comparing the BMIs of male and female cheetahs. Whisker represent maximum and minimum values, while boxes indicate median, 25th and 75th percentiles. Mean values are indicated by the +.

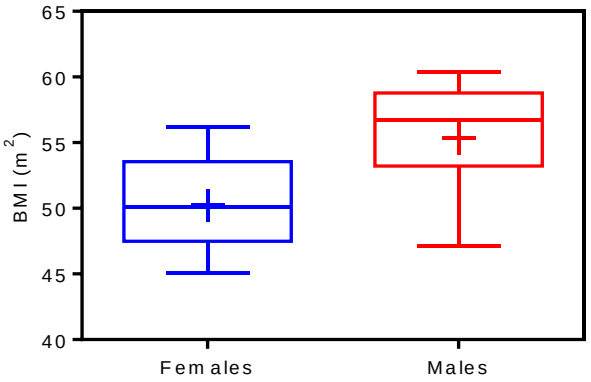


Figure 2-3. Scatterplot of BMI against age in male cheetahs. Linear regression line (Y = -0.06*X + 55.17)

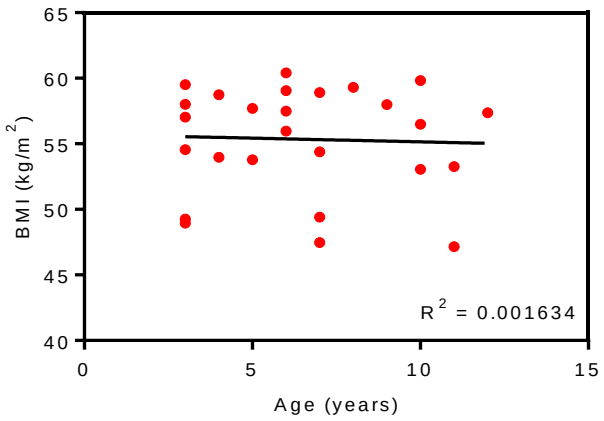
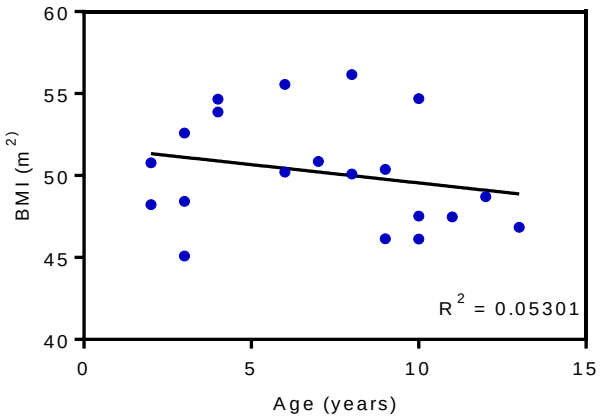
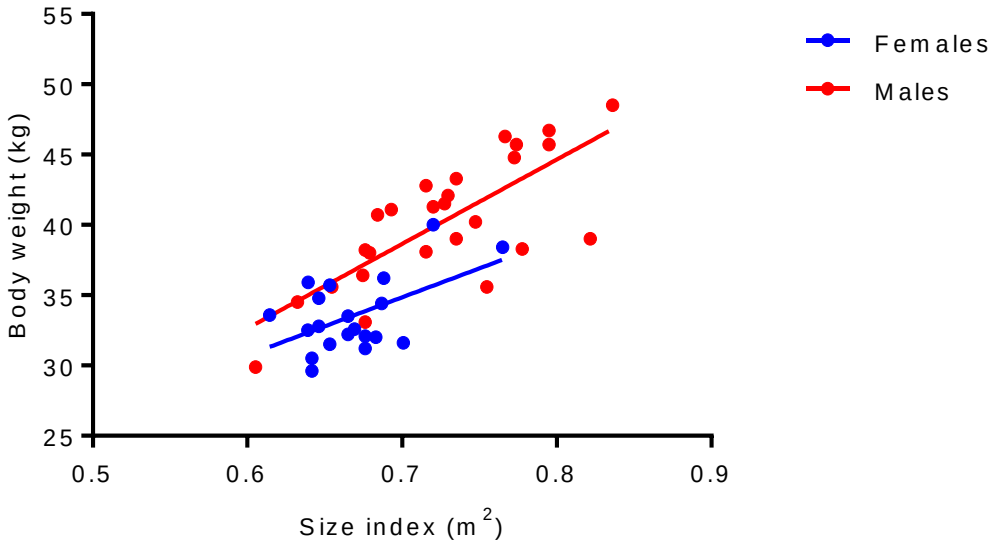


Figure 2-4. Scatterplot of BMI against age in female cheetahs. Linear regression line (Y = -0.22*X + 51.78)



The body weights of males and female cheetahs relative to body size are shown in Figure 2-5. Linear regression lines did not overlap as would be expected with significantly different BMI values, but the slopes of the regression lines did not differ significant between males and females ($F = 0.8$, $p = 0.38$).

Figure 2-5. Scatterplot of body weights relative to size indexes in male and female cheetahs, with linear regression lines for each sex.



2.4 Discussion

Body weights in captive male and female cheetahs were evaluated relative to body size in order to estimate their relative body condition. As expected male cheetahs were significantly larger and heavier than female cheetahs. This sexual dimorphism is consistent with the findings in free-ranging cheetah populations in Namibia (118) and Botswana (123).

The mean body weights of the captive cheetahs in the present study were 5 to 6 kg lower than that of the mean weights in Botswanan cheetahs (males - 46.7 kg, females - 38.5 kg) and 4 to 5 kg less than the mean weights of the Namibian cheetahs (males - 45.6 kg, females – 38.5 kg). The cheetahs in this study were also of shorter body length and had shorter mean shoulder heights (fore limb length), than either the Botswanan cheetahs (males SH – 0.775 m and BL – 1.06 m, females SH 0.733 m and BL – 0.996 m) or the free-ranging Namibian cheetahs (males SH – 0.77 m and BL – 1.021 m, females SH – 0.736 m and BL – 0.983 m). BMI values calculated from the mean data in the free-ranging Namibian (males – 58.0, females – 51.42) and Botswanan cheetahs (males – 56.9, females – 52.74), were only slightly higher than the mean values of 55.35 and 50.22 calculated for males and females respectively in this study. It is possible that these small differences are due to greater muscle mass in the more physically active free-ranging cheetahs. The comparable BMI values indicate that the majority of captive cheetahs in our study are neither overweight nor underweight for their relative body size. The cause of the smaller body size in the captive cheetahs is not clear, but would more likely be due to environmental rather

than genetic factors as these animals came from similar areas as those in the Marker and Dickman study (118) and there are no geographical boundaries separating the Namibian cheetahs from the Botswanan population reported on by Boast *et al* (123). Metabolic bone disease is commonly seen in captive cheetahs due to either calcium or vitamin D_3 deficiencies (124). Although no clinical cases were reported in the captive cheetahs in our study, it is possible that subclinical deficiencies may have resulted in suboptimal growth.

The higher BMI values in male cheetahs indicate that they have a higher body mass to size ratio than females. This is likely to be due to a higher relative muscle mass rather than differences in body fat reserves as the difference is also obvious in free-ranging cheetahs as noted earlier. This is in contrast to lions, where BMI values do not significantly differ between males and females, despite their obvious sexual dimorphism (Tordiffe unpublished data). Although muscle mass differences may be attributed to higher testosterone in males or to higher reproductive metabolic demands in females, both males and females in this study were treated with contraceptives and were therefore downregulated in terms of their reproductive hormones. The differences in BMI are therefore not likely to be influenced by differences in testosterone or reproductive status in adults.

Body mass indices did not appear to be associated in any way with age in either male or female captive cheetahs. A few older females seem to have BMI score that are lower than the mean of the population, but no clear pattern is evident from the data. Many of the chronic diseases seen in captive cheetahs increase in frequency and severity relative to age (9,11). Despite the high incidence of glomerulosclerosis and gastritis recorded in captive cheetahs, weight loss relative to age is not evident in this captive population of cheetahs.

The significant difference in BMI values between males and female cheetahs is of importance in terms of the various metabolite profiles assessed in this study since any relationship between metabolite concentrations and BMI values will have to be evaluated separately for the different sexes. Skeletal muscle and fat are both important tissues from a metabolic point of view and their relatively mass must be considered in any study involving blood or urine metabolite concentrations. In this part of the study, I created a method of approximating body condition in cheetahs by means of a body mass indexing system similar to those used in humans and other species. Body mass indexing methods are limited in that they are unable to distinguish between the weight of fat reserves and skeletal muscle mass. Nevertheless, the cheetah population in this study is expected to be relatively uniform compared to both humans and dogs, where a variety of body types and levels of muscling are evident.

CHAPTER 3
QUANTIFICATION OF URINE METABOLITES

3.1 Introduction

Urine is valuable from an analytical point of view as it contains a large number of metabolites, produced by homeostatic mechanisms within an organism, that are likely to remain relatively unaltered if collected soon after capture and immobilization. The quantification of urinary metabolites is ideally done on the combined urine collected from an animal over a 24-hour period. Such 24-hour samples are impractical for obvious reasons in non-laboratory animals. Urine production may vary between individuals relative to renal function and at different times of the day depending on water intake. Therefore, the concentrations of metabolites in spot or timed urine samples are normally corrected to reflect changes in dilution status. This correction is usually calculated relative to urine creatinine (CR) concentration, urine specific gravity (SG) or urine osmolality. It is argued that since the level of hydration is the largest factor affecting the concentration of urine metabolites, correction of spot urine samples is essential (125-127).

Over the last few decades, CR excretion has most commonly been used to correct urine metabolite concentrations, as this breakdown product of creatine phosphate is produced at a fairly constant rate, is independent of diuresis and is excreted unchanged by the kidneys (128). Metabolite concentrations are typically reported as a ratio of the metabolite concentration to the CR concentration. Creatinine production is thought to be largely reflective of muscle mass, varying somewhat with age, sex, physical exercise and dietary intake in humans (129,130). Concerns have, however, been raised about the universal use of creatinine excretion to correct urine metabolite concentrations (131-133). In most metabolomics studies, urine metabolite adjustments using urine CR concentrations are still the norm, while in occupational toxicology (134,135), endocrinology (136) and drug monitoring (137) either CR or SG corrections are used. Theoretically, CR concentrations should only be used to adjust urine metabolites that are eliminated from the kidneys largely through glomerular filtration without significant tubular reabsorption (127). In metabolomics studies where the urine concentrations of large numbers of metabolites are evaluated, it is generally not possible to selectively adjust each metabolite using a different method, since the mechanisms of elimination are often not known.

Urine SG is a simple, rapid, inexpensive and reliable test to determine urine concentration. It represents the ratio of the density of urine to the density of pure water at the same temperature (138). Unlike osmolality, urine SG is affected by the molecular mass of the particles in solution. Variations in urinary pH, glucose, protein, haemoglobin, bilirubin, ketones and urobilinogen could therefore alter urine SG (139) In healthy animals, however, it has been shown to closely correlate

with osmolality (140) and since urine osmolality is difficult to measure in a clinical setting, SG is far more commonly used to measure urine solute concentration. Specific gravity has been used to correct urine drug concentrations in human subjects (137) and appears to offer some advantages over the CR method of standardization, especially when urine CR values are highly variable (136). When comparing urine metabolites across a population with different variables that are known to impact urine CR concentrations such as sex and age, adjustments using SG may provide more accurate approximations of metabolite excretion (127).

Reference ranges for urine SG or CR concentrations have not been reported in cheetahs. In fact, despite the high incidence of renal disease in this species, urine metabolite, electrolyte or other urine laboratory parameters are rarely mentioned in the literature. In one study a mean urine SG and standard deviation of 1,054 ± 0.006 was reported in 12 captive cheetahs (1.5 to 7.5 years of age) (141), but no other clinical urine parameters have been reported for the species.

3.2 Materials and methods

Once the cheetahs were sufficiently anesthetised as described in Chapter 2, urine was collected within 15 minutes into 5ml cryogenic vials (Corning, USA) after urethral catheterisation using a sterile 6FG dog urinary catheter (Buster ® disposable dog catheter, Kruuse LTD, UK). The urine was frozen immediately at -20°C and kept at this temperature until analysis.

In the laboratory, urine was thawed at room temperature. The CR concentration of each sample was determined by an enzymatic method on an Indiko Clinical Chemistry Analyzer (Thermo Scientific) using the manufacturer's kit and instructions. The enzymatic reaction involves the conversion of creatinine to sarcosine with the aid of creatininase and creatinase. Sarcosine is then converted to glycine, formaldehyde and hydrogen peroxide by sarcosine oxidase. The hydrogen peroxide reacts with 4-aminophenazone and HTIB to form quinone imine chromogen in a reaction catylysed by peroxidase. The colour intensity is directly proportional to the concentration of creatinine. The urine SG was measured with a hand held refractometer (RHC-200), calibrated with distilled water.

Absolute creatinine (ACR) values were corrected relative to urine SG using as described by Miller *et al* (136), where:

$$\textit{Corrected concentration} = \textit{Raw concentration} \times (SG_{target} - 1.0)/(SG_{sample} - 1.0)$$

The *SGtarget* is a population mean SG, which in this study is equal to 1.054.

Data was tested for normality using the D'Agostino and Pearson omnibus (K2) normality test. Pearson correlation coefficients were used to evaluate the relationship between age and urine SG, while Speaman's rho correlation coefficients were used to compare both ACR concentrations to urine SG as well as corrected creatinine (CCR) concentrations to BMI in males and females. Unpaired t-tests were used to compare the BMI and SG values between males and females, while the Mann-Whitney U test was used for comparisons between CCR and ACR in males and females. All analyses were performed with GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, California, USA). All statistical tests were 2-tailed and significance was defined as $P<0.05$ in all cases.

3.3 Results

Urine samples were collected from a total of 58 cheetahs, including 32 males and 26 females with similar age profiles. The urine SG of one female was exceptionally low (1.006) and her values were therefore excluded from further analyses. A summary of the data for the remaining 57 cheetahs is shown in Table 3-1. The urinary ACR concentrations ranged from 12.9 mmol/L to 115.4 mmol/L with a mean of 48.64 mmol/L, while urine SG values ranged from 1.030 to 1.080 with a mean of 1.054. Both ACR and CCR urine concentrations were not significantly different between males and females. Urine SG values were significantly higher in females than in males ($p = 0.0173$), but the mean difference was relatively small (0.007).

Table 3-1. Summary statistical data in males and females cheetahs.

	Males					Females					
	n	**Max**	**Min**	**SD**	**Mean**	**n**	**Max**	**Min**	**SD**	**Mean**	**p-value**
Age (years)	32	14	3	3.31	6.6	25	12	2	3.69	6.7	0.97
BMI	26	60.4	47.16	4.04	55.35	20	56.16	45.09	3.39	50.22	<0.0001*
Absolute creatinine (mmol/L)	32	90.7	12.9	24.2	46.10	25	115.4	13.88	26.72	51.67	0.625
Corrected creatinine (mmol/L)	32	111.4	15.14	23.29	47.51	25	107.7	16.36	24.0	47.54	0.887
Urine specific gravity	32	1.072	1.030	0.010	1.052	25	1.080	1.035	0.012	1.059	0.0173*

Histograms showing the frequency distributions of ACR, CCR and urine SG are shown respectively in Figures 3-1 to 3-3. Mean urine ACR values initially increased relative to age, reaching a peak between three and seven years of age, after which they declined rapidly (Figure 3-4). The CCR concentrations followed a similar pattern, peaking in cheetahs of four to six years, declining gradually thereafter (Figure 3-5).

Figure 3-1. Histogram showing the frequency distribution of absolute urine creatinine concentrations in male and female cheetahs.

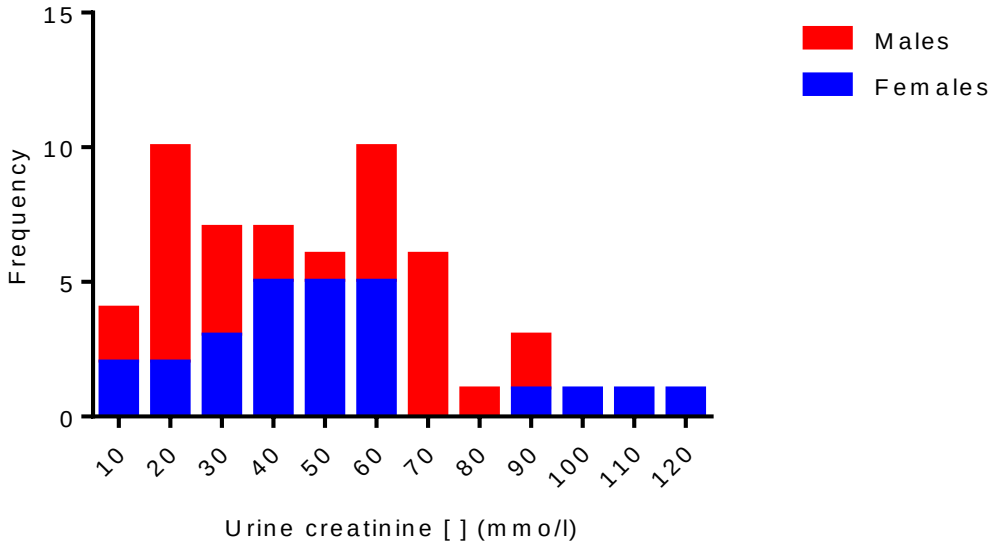


Figure 3-2. Histogram showing the frequency distribution of corrected urine creatinine concentrations in male and female cheetahs

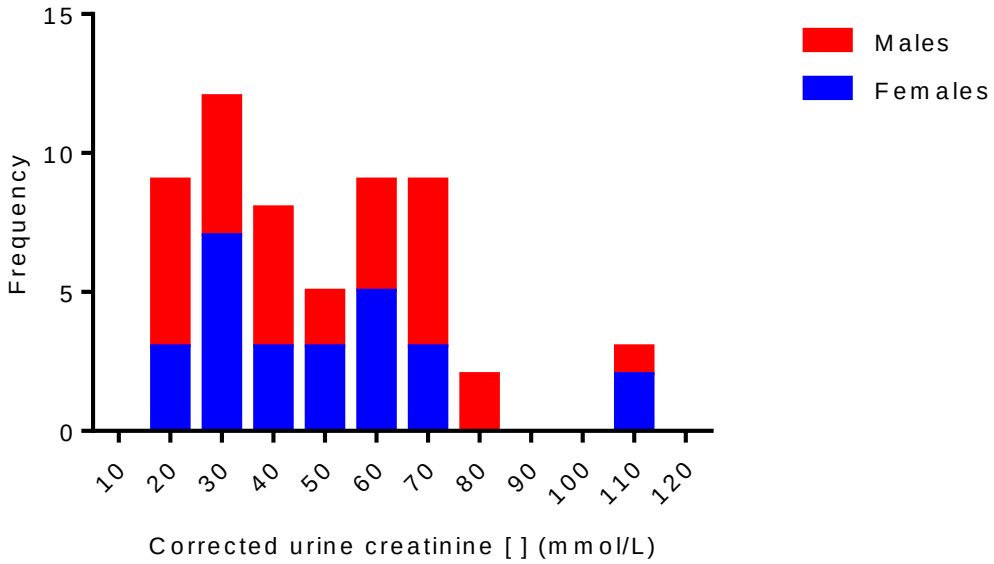


Figure 3-3. Histogram showing the frequency distribution of urine specific gravity values in male and female cheetahs

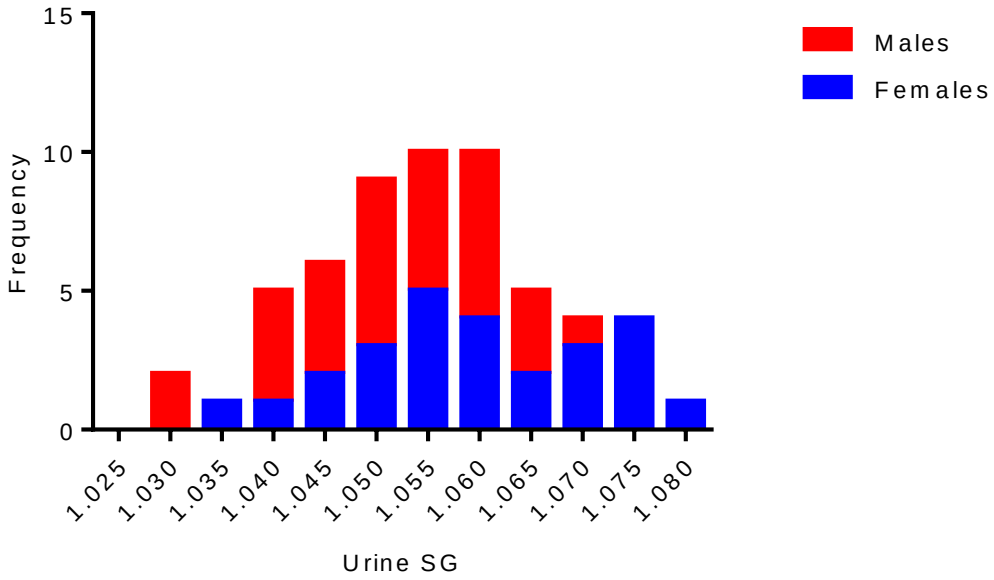


Figure 3-4. Scatterplot of absolute urine creatinine concentrations relative to age in male and female cheetahs.

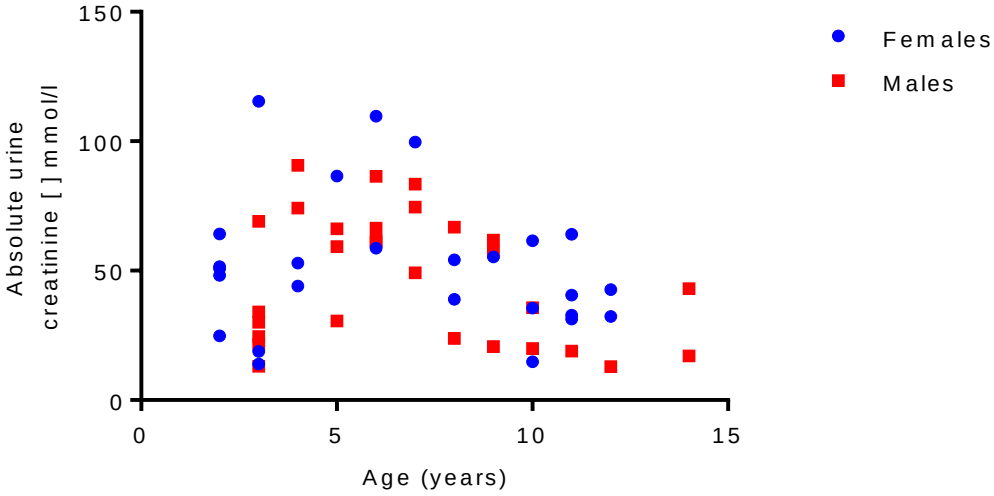


Figure 3-5. Scatterplot of corrected urine creatinine concentrations relative to age in male and female cheetahs.

Absolute creatinine concentrations correlated positively with urine SG values (p = 0.0063), but the relationship, shown in Figure 3-6, was relatively weak (*r* = 0.36). Urine SG remained stable relative to age (*r* = 0.08, p = 0.5407) as shown in Figure 3-7.

Figure 3-6. Scatterplot showing the relationship between absolute urine creatinine concentrations and urine specific gravity.

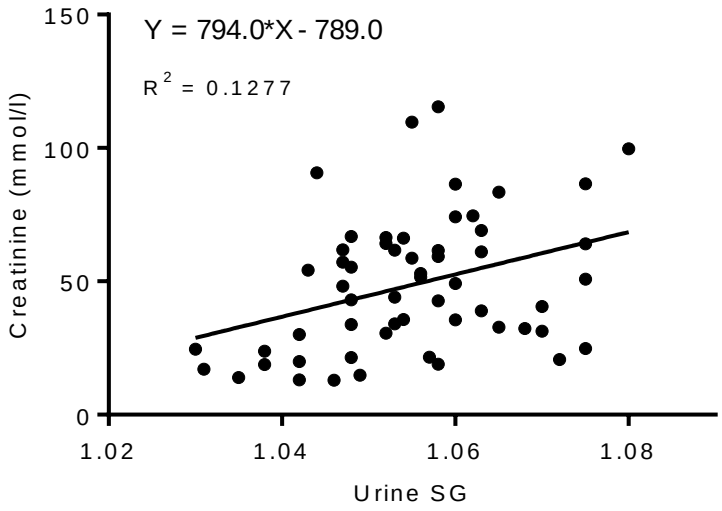
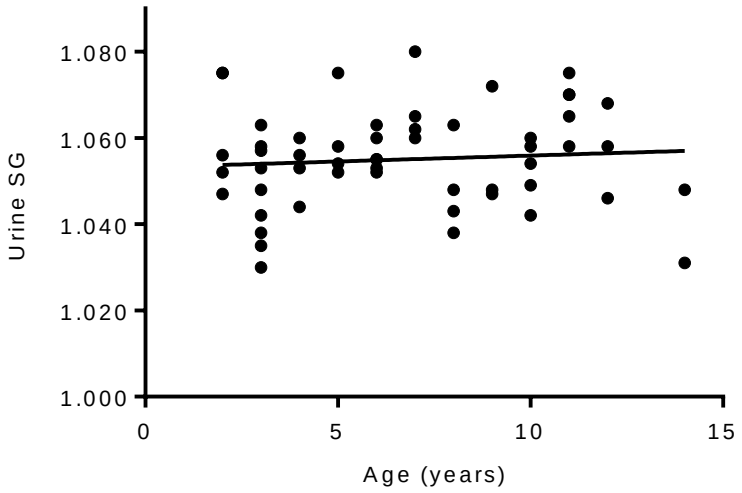
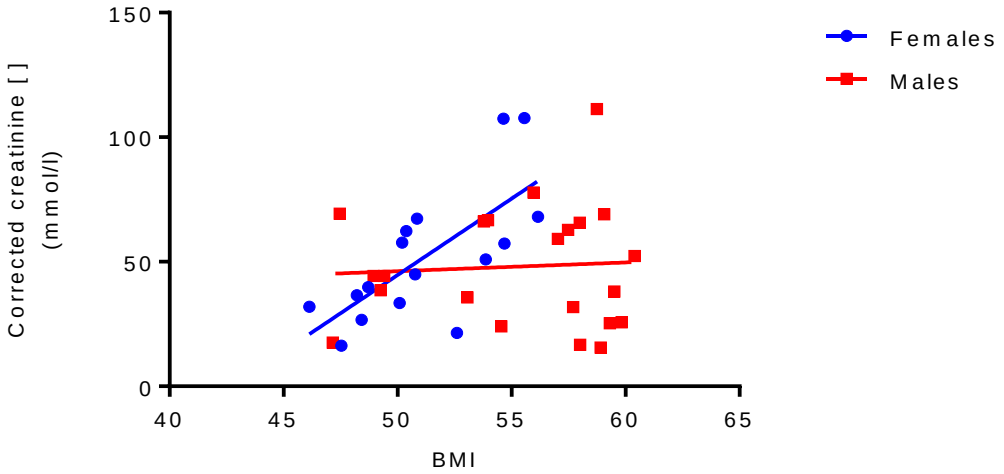


Figure 3-7. Scatterplot showing the relationship between urine specific gravity and age in cheetahs.



A significant positive correlation between urine CCR concentrations and BMI values was found in female cheetahs ($r = 0.7$, $p = 0.0025$), but not in males ($r = 0.06$, $p = 0.78$). The scatterplot showing these differences is shown in Figure 3-8.

Figure 3-8. Scatterplot showing the different relationships between corrected urine creatinine concentrations and body mass index values in male and female cheetahs.



3.4 Discussion

Urine CR concentrations have been used for many years to adjust the concentrations of a range of excreted metabolites, drugs and toxins, where 24-hour urine collection were not feasible. The absolute concentration of the target analyte is simply divided by the urine CR concentration resulting in a corrected concentration expressed per millimole or milligram of CR. It is argued that the calculation is valid since CR is produced and excreted at a constant rate relative to urine production. Yet, this assumption has been questioned as far back as 1958 (142) and by others since then (143-145)

Similar to domestic cats (146), CR excretion in the cheetahs varied tremendously, with more than an eight-fold difference between the minimum and maximum concentrations. This variation must be viewed in light of the fact that water balance (water intake and excretion) in carnivores is very tightly regulated (147) and therefore massive variation in water consumption in cheetahs would not be expected. In addition, the cheetahs in this study received a fairly uniform meat diet and therefore the dietary intake of either creatine, creatine phosphate or creatinine would be expected to be similar between individuals. This is in contrast with human and other animal studies where creatine intake would largely depend on meat consumption in an omnivorous diet, and would therefore be more variable (148). The decrease in CR excretion in older cheetahs is unlikely to reflect a proportional decrease in muscle mass, since BMI scores did not decrease significantly relative to age as shown in the previous chapter. Furthermore, the mean urine CR concentrations were slightly higher in females than in males even though males had significantly higher BMI, and thus presumably a larger proportional muscle mass. The correlation between CCR concentrations and BMI in female cheetahs, and the lack thereof in males, further suggests that there may be sex differences in CR metabolism in this species.

The mean urine ACR concentration recorded in the cheetahs (48.64 mmol/L) in this study was at least three times higher than values typically recorded in human urine (3,6) and three to four-fold higher than values recorded in feral cats on a natural carnivorous diet (12.0 mmol/L in females and 16.2 mmol/L in males) (23). The difference between urine CR concentrations in cheetahs and humans can be partially explained by the fact that cheetah urine is almost three times more concentrated, as reflected by the difference in mean SG values (1.022 in humans (3) versus 1.054 in our cheetah). If the mean human CR concentration is adjusted relative to their mean SG value, using the same cheetah population mean (*SGtarget* = 1.054), then their CCR excretion would be around 35.57 mmol/L [14.49 mmol/L x (1.054-1)/(1.022-1)]. This would mean that the average human excretes approximately 27% less CR than the average cheetah. This difference could further be explained by the likely higher relative lean muscle mass and the higher level of meat consumption in cheetahs compared to humans.

Following a high protein meal, urea production increases dramatically due to the oxidation of amino acids in the urea cycle. In a study on hypercarnivorous mink, Tausen *et al* compared the effects of high and low protein diets (148). They found that on the higher protein diet, 24-hour urine urea excretion was approximately double that of mink on the low protein diet, but urine CR did not differ significantly between the groups. On the high protein diet urinary GFRs were clearly higher, as reflected by a dramatic increase in urine production, yet urine osmolality remained fairly constant. Dietary protein did not significantly influence total water turnover, but on the high protein diet, more water was excreted through the kidneys, while on the low protein diet, water was primarily excreted in the faeces. Similarly, 24-hour urine production decreased from a mean of 95 g/day in fed mink to 25g/day in fasted mink. Although the urine CR concentrations differed significantly, the 24-hour excretion of urinary CR did not change significantly. In domestic cats, protein intake correlated positively with urine SG and urea excretion, and only to a lesser extent with urine CR (146,149). In our study, correlations between urine SG values and ACR concentrations were weak (Figure 3-6), while in studies on omnivores these two parameters are often correlate significantly (137,150). Taken together, this suggests that in carnivores, the concentration of urinary CR is poorly correlated to urine production and therefore provides an inadequate reflection of GFR. The rate of CR production in the body is clearly a distinctive process from the rate of urinary CR excretion through the kidneys, yet both variables could potentially influence the final CR concentration measured in a spot urine sample. Holder *et al* found that endogenous creatinine clearance correlated well with urinary clearance of inulin in anaesthetised cheetahs ($R^2 = 0.928$, $p < 0.0001$), and since inulin excretion is considered the gold standard for GFR determination, CR clearance was shown to provide a very accurate approximation of GFR in cheetahs (141). This merely indicates that CR is excreted at a rate that is proportional to GFR, but does not offer any indication of the consistency of CR production in the muscles of this species. It seems unlikely that GFR would vary eight-fold, in line with urine CR concentrations in our cheetahs, since only a three-fold difference has been demonstrated between the GFR in healthy domestic cats and the GFR in those with chronic renal failure (151). Therefore, in the absence of obvious renal failure, the high variability in urine CR concentrations in the cheetahs in our study can only be explained by individual variation in muscle CR production.

In contrast to CR concentrations, we found only a 2.7-fold difference between the highest and lowest urine SG values in this cheetah population. In carnivores, urea accounts for about 80% of the urine solutes excreted and therefore has the largest single impact on urine osmolality. Since urea is a small molecule (molar mass = 60.06 g/mol), it has a comparable influence on urine osmolality and urine SG. Thus, in healthy carnivores where urine is relatively free of glucose, haemoglobin, bilirubin and other larger molecules, urine SG would be expected to provide a good approximation of urine osmolality. In cheetahs that receive a diet with fairly consistent levels of

protein and where fasting is applied for the same period of time in all individuals prior to sample collection, one would expect that urine production would not vary a great deal in the sampled population. Corrected metabolite concentrations would therefore be expected to be only moderately different to absolute values. Inter-individual variations in water intake would be small, as has been observed in other carnivores, and should be reflected by changes in urine SG. Thus, we would expect SG correction of urine metabolite concentration to be a more accurate reflection of 24-hour metabolite excretion in cheetahs.

In these cheetahs, urine SG values did not change significantly relative to age (Figure 3-7). The oldest cheetahs in this study were however only 14 years old, and it is possible that a decline in urine SG would only be seen in more aged animals as noted in both humans (152) and dogs (153). As with many other biomarkers, urine SG is a poor indicator of early renal failure and therefore it is not possible to determine the incidence of subclinical renal disease in this population. However, the renal concentrating ability in these cheetahs was clearly not compromised. One cheetah with a urine SG value of 1.006 may have been suffering from chronic renal failure, but this animal was excluded from the study.

In conclusion, CR excretion in this population of cheetahs was highly variable and appears to be influenced by individual, largely age-related differences in CR production rather than accurately reflecting changes in glomerular filtration rate. Relying on urine CR to correct or “standardize” urine metabolite concentrations would therefore result in the possible overestimation of metabolite concentrations in younger and older cheetahs. Variability in urine SG was considerably lower, as would be expected in a carnivorous mammal where water balance is tightly regulated. In cheetahs, where dietary protein intake is fairly similar between individuals, and where they are starved for the same period of time prior to sample collection, urine SG is likely to provide a better indication of urine dilution. It therefore, in our view, would provide a more accurate means of correcting urine metabolite concentrations. Furthermore, the age-related reduction in CR production is interesting and warrants future research to elucidate both the cause and physiological impact of this decline.

CHAPTER 4
URINE ORGANIC ACIDS

4.1 Introduction

Urinary organic acids are a broad class of water-soluble end-products and intermediates of a wide range of metabolic pathways, including the metabolism of amino acids, carbohydrates, fatty acids, ketones and biogenic amines. The quantification of urinary organic acids has primarily been used in the detection and management of inborn errors of metabolism (154). Changes in the urinary excretion of organic acids have, however, also provided valuable information for the early diagnosis of metabolic (155,156) and neurological disorders (157,158) and have broadened our understanding of the metabolic alterations associated with intestinal dysbiosis (159), allergies (160) and malnutrition in humans (161,162).

Broad studies evaluating the urine organic acids in non-laboratory animals are extremely limited. Metabolite fingerprinting of urine in domestic dogs has been used to demonstrate metabolic and nutritional differences between breeds (91), and the urine metabolite changes in aging and calorie-restricted dogs have also been studied using nuclear magnetic spectroscopy (163). In general, however, research on the volatile organic acids of urine in both domestic and wild felids have been limited to the study of metabolites that are considered important in territorial marking and other behaviours (101,164).

In the present study we document the organic acid metabolites in urine from cheetahs using gas chromatography-mass spectrometry (GC-MS) in order to develop a basic understanding of their metabolism, and generate new hypotheses for further investigations into the mechanisms of diseases they suffer from in captivity.

4.2 Materials and methods

4.2.1 Sample collection

Urine was collected from 57 apparently healthy adult cheetahs as described in Chapter 3. Ages ranged from 2 to 14 years (males = 32 and females = 25) (Table 4-1). The majority of the samples (n = 40) were collected from captive cheetahs housed at the AfriCat Carnivore Care Centre near Otjiwarongo in Namibia during routine annual health examinations. Additional samples were obtained from two 7-year-old free-ranging cheetahs at the Okonjima Reserve adjacent to AfriCat for comparison. These animals were well habituated to humans and were easily immobilized via

remote injection (darting) in the same way as the captive cheetahs. The remaining 15 samples were collected from cheetahs housed at various facilities of the National Zoological Gardens of South Africa (NZG). Unlike the AfriCat cheetahs, the NZG animals were born in captivity, housed in smaller zoo type enclosures (100 – 250 m^2) and fed a diet of whole eviscerated chicken or beef, supplemented with a vitamin and mineral powder (Predator®, Healthtech Laboratories, South Africa).

Once sufficiently anaesthetised, urine was collected from the cheetahs within 15 minutes into 5ml cryogenic vials (Corning, USA) after urethral catheterisation using a sterile 6FG dog urinary catheter (Buster® disposable dog catheter, Kruuse LTD, UK). The urine was frozen immediately at -20°C and kept at this temperature until analysis.

4.2.2 Urine specific gravity and creatinine determination

The urine was thawed at room temperature. The urine specific gravity (SG) was measured with a hand held refractometer (RHC-200), calibrated with distilled water. The creatinine concentration of each sample was determined by an enzymatic method on an Indiko Clinical Chemistry Analyzer (Thermo Scientific) using the manufacturer's kit and instructions.

4.2.3 Organic acid analysis

The methodology for the organic acid analysis was described by Rinaldo 2008 (165) and subsequently modified and performed as described by Reinecke et al (166). In short, 100 µl of the internal standard (3-phenylbutyric acid, 52.5mg/dl, Sigma Chemical Company) was added to 1ml of urine and acidified to a pH < 2 with 5N HCl. The organic acids were extracted with 5 ml ethyl acetate and 3 ml diethyl ether consecutively by shaking for 15 minutes. After centrifugation, the organic phase was removed and pooled in a 10 ml Kimax tube dried with sodium sulphate, centrifuged again and transferred to a second clean Kimax tube, which was then dried under nitrogen. The dried organic acids were derivatized with O-bis(trimethylsilyl)trifluoroacetamide (BSTFA): trimethylchlorosilane (TMCS): pyridine (100:20:20 µl), for 45 minutes at 85 °C and 1 µl was injected into the GC-MS.

The Agilent GC–MS system used in this study consisted of a model 7890A gas chromatograph, a model 5975C mass selective detector, an HP 5970 C MS and Agilent Chemstation (Revision E.02.00). A fused-silica capillary column (DB-1MS UI, 30 m, 2.50 lm i.d., 0.25 lm film thickness) was used for the fractionation. The initial GC temperature was kept at 60°C 2 min. It was then

increased to 120°C at a rate of 5°C /min, to 295°C at a rate of 7°C /min, and then held at a final temperature of 295 °C for 2 min. Helium (1 ml/min) was used as a carrier gas at a constant flow rate. The mass spectra of all GC peaks were generated by a mass spectrometer operated at 70 eV in the electron impact mode with SCAN (50–600 amu) positive ion monitoring. The MS source and quadrupole temperatures were 230 and 150°C respectively.

The deconvolution and data analysis were performed using AMDIS software (Version 2.66) linked to the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.0g, built May. 19, 2011). An AMDIS library file, consisting of more than 800 mass spectra, was generated from the NIST/EPA/NIH Mass Spectral Library, as well as from the Wiley Registry™ of Mass Spectral Data (8th Edition). Mass spectra of unknown compounds present in relatively high concentrations were added to the target file. Where available, mass spectra were confirmed by analysing authentic standards.

The Potchefstroom laboratory for Inborn Errors of Metabolism (PLIEM), where the analyses were done, participates in an external quality control program, Quality Assurance In Laboratory Testing For IEM (ERNDIMQA) http://www.erndimqa.nl/ . All quality control was done according to the specification of ERNDIMQA on material supplied by ERNDIMQA (Control Organic Acids, Product code- ORG-01, Batch number- 2016.007 (http://cms.erndimqa.nl/getdoc/440cb2de-9489-4e46-8477-84be0a9309bb/2016-007-PC-Org-Acids_v1.aspx). However, due to the possible presence of hundreds of organic acids in urine and unavailability of standards for all the organic acids, a response factor of 1 was used to quantify organic acids that were not present in the ERNDIMQA sample material. The calculated concentrations (Calculated concentration = Area of metabolites/Area of internal standard x concentration of internal standard x response factor) were therefore relative to the response and concentration of the internal standard.

Corrected metabolite concentrations were calculated relative to urine specific gravity (SG) as described by Miller et al (136), where:

Corrected concentration = Raw concentration x (SGtarget -1.0)/SGsample – 1.0)

The *SGtarget* is a population mean SG, which in this study is equal to 1,054.

A data matrix was created by aligning all the metabolites against the samples using MATLAB (167) and IBM SPSS Statistics Version 22 (168) software.

4.2.4 Identification and synthesis of unknown metabolites

Tentative identification of metabolites was made by spectral comparison using the NIST. Mass Spectral Search Program (Version 2.0.g). The definitive identification of unknown compounds was achieved by comparison to synthetically produced metabolite standards. N-Acetylglycine (N-Acylamino acid) conjugates were prepared using the method described by Furniss *et al* (168). The acyl chloride component of the organic acids (benzoate, phenylacetate, phenylpropionate, 4-hydroxyphenylacetate, 4-hydroxyphenylpriopionate and 4-hydroxyphenylacrylate) was prepared with thionyl chloride as described by the same authors for cyclohexanecarbonylchloride. The organic acid (0.5 mol) was placed in a two-necked flask fitted with a dropping funnel and reflux condenser. The flask was heated over a water bath and redistilled thionylchloride (0.6 mol) was added over a period of 45 minutes. The flask was shaken from time to time to ensure mixing. The mixture was refluxed for 2 hours and the product was isolated with distillation through a Vigreux column. The N-acylglycine/glutamate conjugate was prepared by dissolving 0.5g of the amino acid in 10ml (10 ml) (10%) sodium bicarbonate to which 1 gram of acyl chloride was added. The mixture was then mixed on a rotary wheel for an hour. The product was extracted with ethyl acetate after lowering the pH to < 2 with 6 N HCl, dried, derivitized with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and T Trimethylchlorosilane (TMCS) and then analysed using GC-MS.

4.2.5 Statistical analysis

All results are reported as mean concentrations and standard deviations. Spearman's rank correlation coefficients (r) and p-values were calculated for organic acid concentrations relative to age. Correlations were deemed important if $|r| \geq 0.3$ and $p \leq 0.05$. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed to compare the organic acid concentrations of male versus female cheetahs after auto scaling. PCA is an unsupervised (i.e. using no group information to explain observed variation) method used here to assess the generalizability of the supervised PLS-DA model. All analyses were done using IBM SPSS Statistics Version 22 (168) or MetaboAnalyst 3.0 (www.metaboanalyst.ca) software (169).

4.3 Results

A summary of the biometric, urine creatinine and urine specific gravity data for the 57 cheetahs sampled, is shown in Table 4-1. A total of 339 different organic acids could be annotated and quantified from the GC-MS data. Of these metabolites 149 were not detectable in more than 80% of the samples and were therefore excluded, leaving 190 variables for statistical analysis. Although urea was detected in abundance in the urine samples, the quantification of urea by GC-MS is considered to be unreliable and it was therefore excluded from the analysis (170). Representative chromatograms of the urinary organic acids of both the wild and captive cheetahs are shown in Figures 4-1 and 4-2 respectively. The mean concentrations of the thirty most abundant organic acids are listed in Table 4-2. The most abundant organic acids in the captive cheetahs' urine were an unidentified compound with a total molecular mass of 362 (annotated Unknown 362) and a compound tentatively identified as N^1,N^5-dimethylpentane-1,5-diamine. A number of glycine conjugates, were detected at relatively high concentrations. Krebs cycle intermediates, intermediate products of fatty acid metabolism and metabolites formed by the degradation of particularly the aromatic amino acids were also abundant.

Table 4-1. Comparative biometric, urine creatinine and urine specific gravity data for the study group of male and female cheetahs

	Males					Females					
	n	**Max**	**Min**	**SD**	**Mean**	**n**	**Max**	**Min**	**SD**	**Mean**	**p-value**
Age (years)	32	14	3	3.31	6.6	25	12	2	3.69	6.7	0.97
BW (kg)	26	48.5	29.9	4.6	40.25	21	40.0	29.6	2.64	35.56	<0,0001*
Size Index (m^2)	26	0.836	0.605	0.06	0.73	20	0.765	0.614	0.03	0.67	0.0002*
BMI	26	60.4	47.16	4.04	55.35	20	56.16	45.09	3.39	50.22	<0.0001*
ACR (mmol/L)	32	90.7	12.9	24.2	46.10	25	115.4	13.88	26.72	51.67	0.625
CCR (mmol/L)	32	111.4	15.14	23.29	47.51	25	107.7	16.36	24.0	47.54	0.887
Urine SG	32	1.072	1.030	0.010	1.052	25	1.080	1.035	0.012	1.059	0.0173*

Table 4-2. Mean concentrations (mmol/L) and standard deviation (SD) of the 30 most abundant organic acids detected in cheetah urine.

Metabolite	*Mean (SD)*	*Metabolic origin*
Unknown 362	466.76 (375.27)	Unknown
N^1,N^5-Dimethylpentane-1,5-diamine	268.50 (197.81)	Lysine
N-Phenylacetylglycine	111.00 (47.23)	Enteric bacteria/Phenylalanine
Hippuric acid	76.62 (37.10)	Enteric bacteria/Phenylalanine
4-Hydroxyphenylacetic acid	59.58 (20.04)	Enteric bacterial/Tyrosine
Oxalic acid	45.16 (29.21)	Glycine metabolism
Aconitic acid	43.81 (18.00)	Krebs cycle intermediate
2-Hexenoic acid	29.12 (30.51)	Fatty acid metabolism
Citric acid	26.47 (31.02)	Krebs cycle intermediate
Lactic acid	25.48 (15.79)	Glycolysis
Isocitric acid	19.43 (11.06)	Krebs cycle intermediate
4-Hydrocinnamic acid	17.75 (25.52)	Enteric bacteria/Phenylalanine
Phosphoric acid	17.06 (25.63)	Phosphate
2,3-Dihydroxybutanoic acid	14.06 (6.92)	Carbohydrate metabolism
N-Phenylpropionylglycine	13.17 (21.46)	Enteric bacteria/Phenylalanine
N-4-Hydroxyphenylacetylglycine	12.42 (6.62)	Enteric bacteria/Tyrosine
Cinnamoylglycine	11.11 (8.73)	Enteric bacteria/Phenylalanine
4-Hydroxymandelic acid	9.43 (17.15)	Enteric bacteria/Tyrosine
N-Isovalerylglycine	8.43 (5.60)	Leucine metabolism
N-Hexanoylglycine	7.93 (5.44)	Fatty acid metabolism
1,2-Dihydroxycyclohexene	7.69 (15.47)	Unknown
Succinic acid	7.66 (3.95)	Krebs cycle intermediate
Methylsuccinic acid	7.13 (6.81)	Fatty acid metabolism
3-Methylglutaconic-acid	7.02 (14.19)	Leucine metabolism
N-4-Hydroxyphenylpropionylglycine	6.02 (8.70)	Enteric bacteria/Tyrosine
2-Hydroxyphenylpropionic acid	5.19 (2.57)	Enteric bacteria/Tyrosine
4-Hydroxyphenyllactic acid	4.94 (2.81)	Enteric bacteria/Tyrosine
N-2-Methylbutyrylglycine	4.80 (4.91)	Fatty acid metabolism
4-Methylpimelic acid	4.51 (3.02)	Fatty acid metabolism
Tricarballylic acid	6.04 (27.19)	Enteric bacteria

Figure 4-1. Annotated chromatogram of the organic acids detected in a free-ranging cheetah.

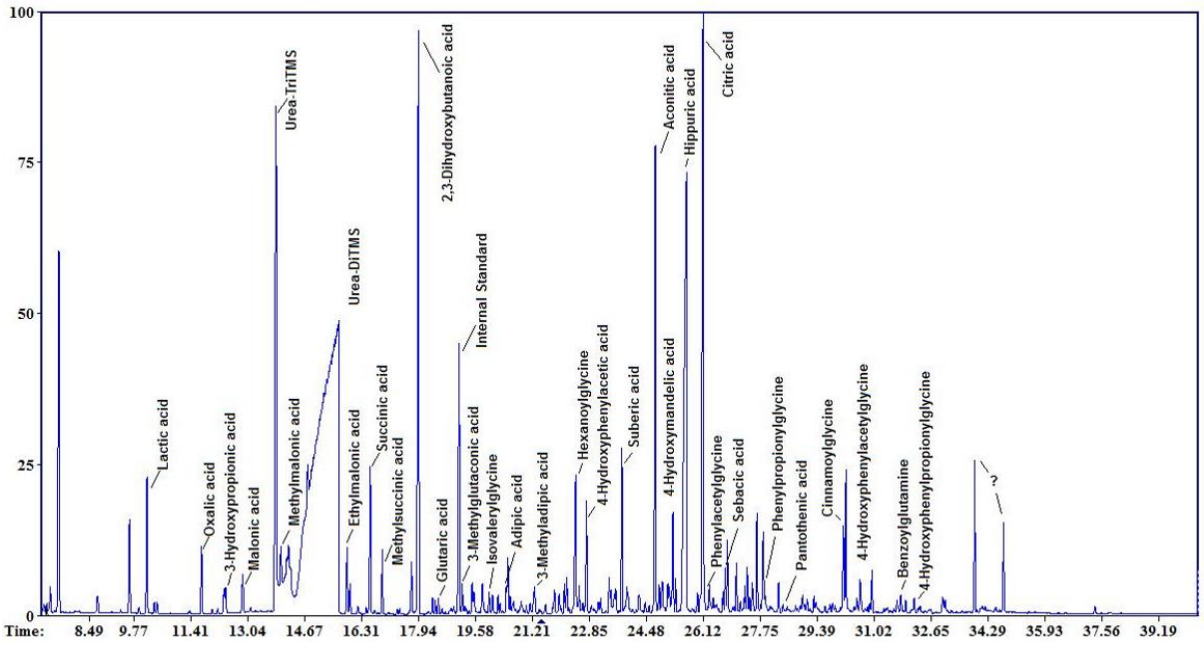
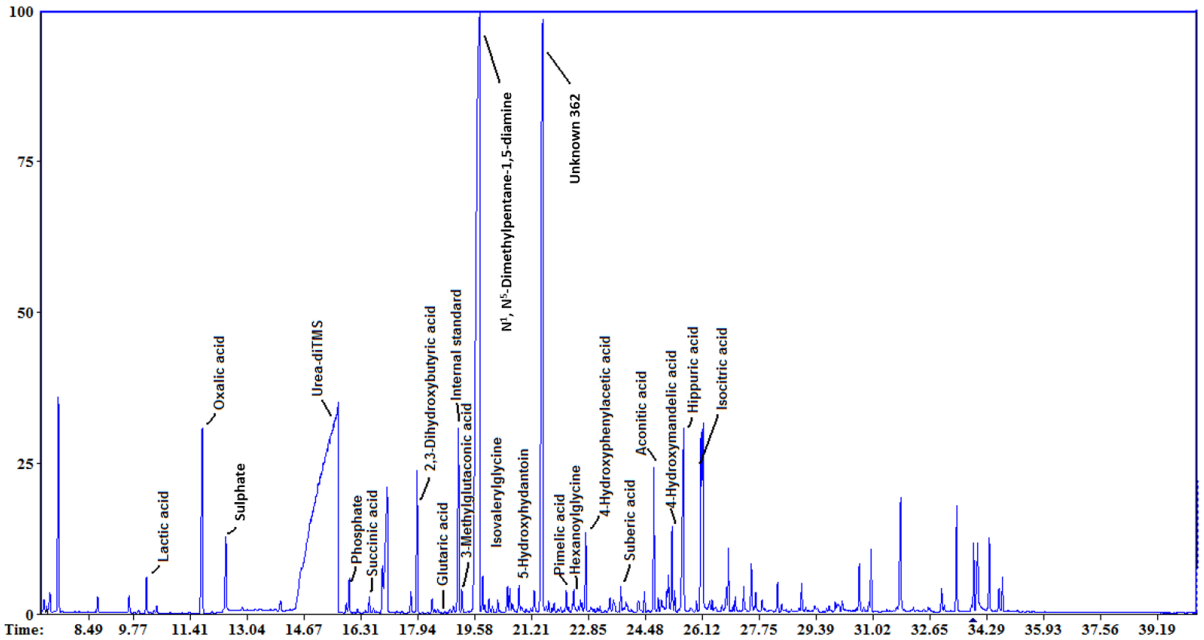


Figure 4-2. Annotated chromatogram of the organic acids detected in the urine of a captive cheetah.



Excretion of several metabolites including pantothenic acid, citramalic acid, 2 deoxy-3,5-dihydroxypentonic acid-γ-lactone, N-acetylisoleucine, ribose, 3-hydroxyphenylacetic acid and vanillylmandelic acid decreased significantly relative to age (based on correlation analysis using

Spearman's rho), while glutaric acid, 2-hydroxyundecanoic acid and 1,2-dihydroxybenzene excretion showed a significant positive relationship with age (Table 4-3).

Table 4-3. Urine organic acid concentrations that correlated with age (in years) in cheetahs (n = 57).

Metabolite	*Metabolic pathway*	*Spearman (r)*	*p-value*
Pantothenic acid	B-vitamin metabolism	-0.408	0.001
Citramalic acid	Enteric bacteria	-0.393	0.002
Glutaric acid	Fatty acid metabolism	0.385	0.003
2-Deoxy-3,5-dihydroxypentonic acid-γ-lactone	Carbohydrate metabolism	-0.383	0.003
N-Acetylisoleucine	Isoleucine metabolism	-0.357	0.006
2-Hydroxyundecanoic acid	Fatty acid metabolism	0.356	0.006
Ribose	Carbohydrate metabolism	-0.348	0.007
3-Hydroxyphenylacetic acid	Enteric bacteria/Phenylalanine	-0.346	0.008
Vanillylmandelic acid	Catecholamine metabolism	-0.336	0.010
1,2-Dihydroxybenzene (Catechol)	Enteric bacteria	0.330	0.011
Isocitric acid	Krebs cycle intermediate	-0.328	0.012
2,3,4-Trihydroxybutyric acid	Carbohydrate metabolism	-0.322	0.014
3-Hydroxysebacic acid	Fatty acid metabolism	-0.321	0.014
Glyceric acid	Glycine metabolism	-0.320	0.014
Ribonic acid	Carbohydrate metabolism	-0.306	0.019
3,4-Dihydroxy-3,7,11-trimethyldodecanoic acid	Co-enzyme Q10 metabolism	-0.303	0.021
Orotic acid	Pyrimidine metabolism	-0.302	0.021
3-Hydroxybenzoic acid	Enteric bacteria	-0.300	0.022

Due to significant differences in BMI values obtained for male and female cheetahs, correlations between BMI values and urine organic acid concentrations had to be evaluated separately for the sexes. In female cheetahs, higher BMI scores were associated with more phenylacetic acid, 4-hydroxycinnamic acid, hippuric acid, 1,4-dihydroxybutane, citramalic acid, 2methyl-1,3-dihydroxybutane, levulinic acid, 5-methyldihydropyrimidine-2,4(1H,3H)-dione, orotic acid, octenedioic acid, galactonic acid and N-acetylisoleucine excretion . Only the excretion of 1,2-dihydroxypropane was determined to decrease with increasing BMI scores in the females (Table 4-4). In male cheetahs, galactonic acid and N-isobutyrylglycine correlated positively with BMI, while 2-hydroxyglutaric acid, 3,4-dihydroxyphenylpropionic acid, 2,4-dihydroxybenzoic acid and 2-hydroxy-3-methylvaleric acid correlated negatively with their BMI scores (Table 4-5).

Table 4-4. Urine organic acid concentrations that correlated with Body Mass Index values in female cheetahs (n=20).

Metabolite	*Metabolic origin*	*Spearman (r)*	*p-value*
Phenylacetic acid	Enteric bacteria/Phenylalanine	0.669	0.003
4-Hydroxycinnamic acid	Enteric bacteria/Tyrosine	0.638	0.006
Hippuric acid	Enteric bacteria/Phenylalanine	0.578	0.015
1,4-Dihydroxybutane	Carbohydrate metabolism	0.556	0.020
Citramalic acid	Enteric bacteria	0.529	0.029
2-Methyl-1,3-dihydroxybutane	Carbohydrate metabolism	0.528	0.029
Levulinic acid	Carbohydrate metabolism	0.517	0.034
5-Methyldihydropyrimidine-2,4(1H,3H)-dione	Pyruvate + Urea artifact	0.508	0.037
1,2-Dihydroxypropane	Carbohydrate metabolism	-0.500	0.041
Orotic acid	Pyrimidine metabolism	0.498	0.042
Octenedioic acid	Fatty acid metabolism	0.490	0.046
Galactonic acid	Carbohydrate metabolism	0.487	0.048
N-Acetylisoleucine	Isoleucine metabolism	0.484	0.049

Table 4-5. Urine organic acid concentrations that correlated with Body Mass Index values in male cheetahs (n=26).

Metabolite	*Metabolic pathway*	*Spearman (r)*	*p-value*
Galactonic acid	Carbohydrate metabolism	0.651	0.001
N-Isobutyrylglycine	Valine metabolism	0.456	0.033
3,4-Dihydroxyphenylpropionic acid	Enteric bacteria/Phenylalanine	-0.453	0.034
2,4-Dihydroxybenzoic acid	Enteric bacteria	-0.443	0.039
2-Hydroxy-3-methylvaleric acid	Isoleucine metabolism	-0.428	0.047

The partial least squares discriminant analysis (PLS-DA) as well as the principal component analysis (PCA) illustrated in Figure 4-3, show clear separation of the wild cheetah profiles from those of the captive animals. In both analyses, the wild cheetah profiles cluster very close together, despite the one being female and the other male. The PCA as well as PLS-DA provided a ranking of important metabolites based on power and variable importance in projection (VIP) values respectively. A list of 22 metabolites (Table 4-6) had power values above 0.5 or VIP values above 3 indicating their importance in differentiating the wild cheetahs from those in captivity. A number of products of fatty acid metabolism (hydroxypyruvic acid, 3-hydroxysebacic acid, undecanedioic acid, tetradecanedioic acid, 4,7-decadienedioic acid, monopentadecanoylglycerol, 2-hydroxysebacic acid, 3,6-dimethylsuberic acid, 4-decenedioic acid, sebacic acid, 2-hydroxyvaleric acid, 3-hydroxyadipyl-γ-lactone, N-valerylglycine, 2,3-dihydroxybutanoic acid, 4-methylpimelic acid and monohexadecanoylglycerol) as well as isoleucine (2-methyl-2-hydroxybutyric acid and 2-hydroxy-3-methylbutryic acid) and threonine (2,3-dihydroxybutanoic acid) metabolites were excreted at higher concentrations in the two wild cheetahs than in their

captive counterparts, while oxalic acid, Unknown 362, N^1,N^5-dimethylpentane-1,5-diamine and N-phenylacetylglycine were detected at significantly higher concentrations in the captive cheetahs.

Figure 4-3. Three dimensional PCA (left) and PLS-DA (right) score plots of urinary organic acid profiles in captive and free-ranging cheetahs

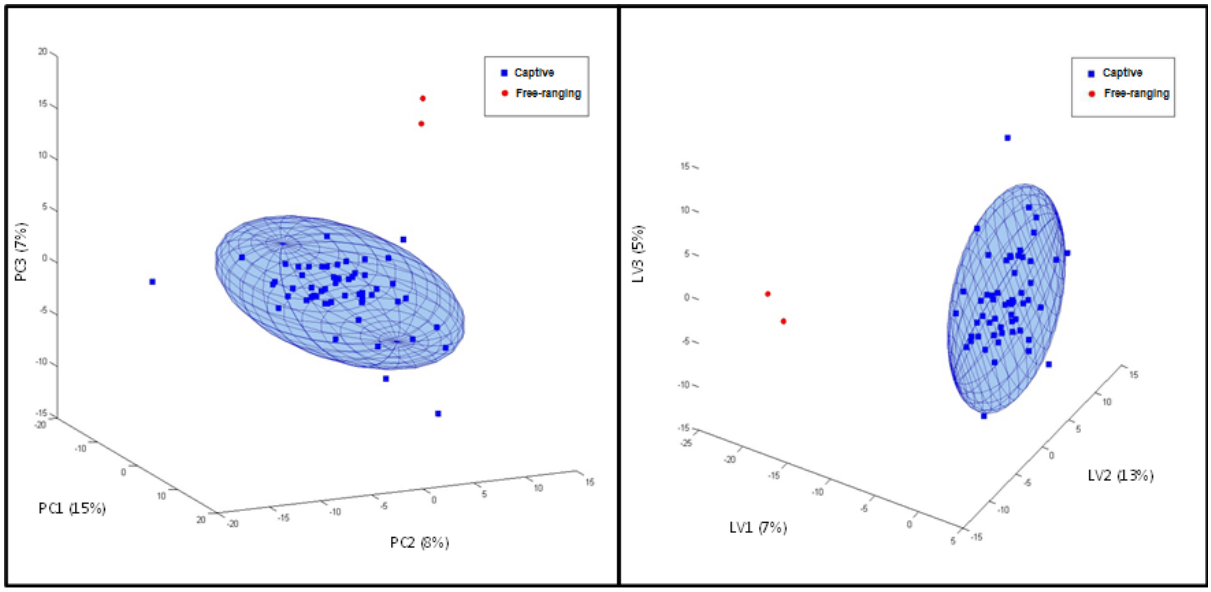


Table 4-6. Multivariate and univariate statistics for potential discriminatory variables comparing urine organic acids in wild cheetahs with those in captivity.

Metabolites	*Wild cheetah mean (mmol/L) (n = 2)*	*Captive cheetah mean (mmol/L) (n = 56)*	*PLS VIP scores*	*Effect Size (r-value)*	*Mann-Whitney U (p-value)*	*Fold change*	*Metabolic origin*
Hydroxypyruvic acid	0.967	0.013	16.205	0.4034	0.0021	-74.80	Glycine,serine,threonine
3-Hydroxysebacic acid	1.617	0.093	13.808	0.3145	0.0166	-17.37	Fatty acid metabolism
Undecanedioic acid	0.546	0.013	12.611	0.4928	0.0002	-41.24	Fatty acid metabolism
Tetradecanedioic acid	0.371	0.012	10.799	0.3872	0.0032	-31.47	Fatty acid metabolism
2-Methyl-2-Hydroxybutyric acid	2.864	0.129	10.611	0.3788	0.0039	-22.13	Isoleucine metabolism
4,7-Decadienedioic acid	0.237	0.008	10.122	0.5875	7.66E-06	-28.81	Fatty acid metabolism
Monopentadecanoylglycerol	2.466	0.068	9.513	0.3412	0.009	-36.08	Fatty acid metabolism
2-Hydroxysebacic acid	0.795	0.052	9.410	0.3314	0.012	-15.22	Fatty acid metabolism
3,6-Dimethylsuberic acid	0.150	0.009	8.208	0.5180	7.97E-05	-17.29	Fatty acid metabolism
2-Hydroxy-3-Methylbutryic acid	1.154	0.082	7.372	0.3663	0.005	-14.04	Isoleucine metabolism
4-Decenedioic acid	2.362	0.297	7.053	0.3238	0.014	-7.95	Fatty acid metabolism
Sebacic acid	4.154	0.264	6.076	0.4751	0.0003	-15.75	Fatty acid metabolism
Oxalic acid	13.216	54.860	5.914	0.3106	0.018	4.15	Glycine metabolism
Unknown 362	0.138	448.17	5.653	0.3105	0.009	3254.10	Unknown
2-Hydroxyvaleric acid	0.285	0.041	5.466	0.3515	0.007	-7.02	Fatty acid metabolism
N^1,N^5-Dimethylpentane-1,5-diamine	0.832	273.283	5.336	0.3106	0.018	328.46	Unknown
N-Phenylacetylglycine	23.389	122.365	4.811	0.3106	0.018	5.23	Enteric bacteria/Phenylalanine
3-Hydroxyadipyl-γ-lactone	0.845	0.204	4.292	0.3007	0.022	-4.15	Fatty acid metabolism
N-Valerylglycine	0.801	0.091	4.020	0.4070	0.002	-8.78	Fatty acid metabolism
2,3-Dihydroxybutanoic acid	71.253	18.138	3.733	0.3106	0.018	-3.98	Threonine metabolism
4-Methylpimelic acid	19.696	4.477	3.323	0.3107	0.018	-4.40	Fatty acid metabolism
Monohexadecanoylglycerol	0.103	0.018	3.000	0.3659	0.005	-5.60	Fatty acid metabolism

Comparison of urine organic acid profiles between male and female cheetahs resulted in separation of the groups by the PLS-DA, but no separation in the PCA (Figure 4-4). A list of seven metabolites responsible for the separation, with PLS-DA VIP scores >3.0, Mann-Whitney p<0.05 and effect size >0.3 was generated (Table 4-7).

Figure 4-4. Three dimensional PCA (left) and PLS-DA (right) score plots of urinary organic acid profiles in male and female cheetahs.

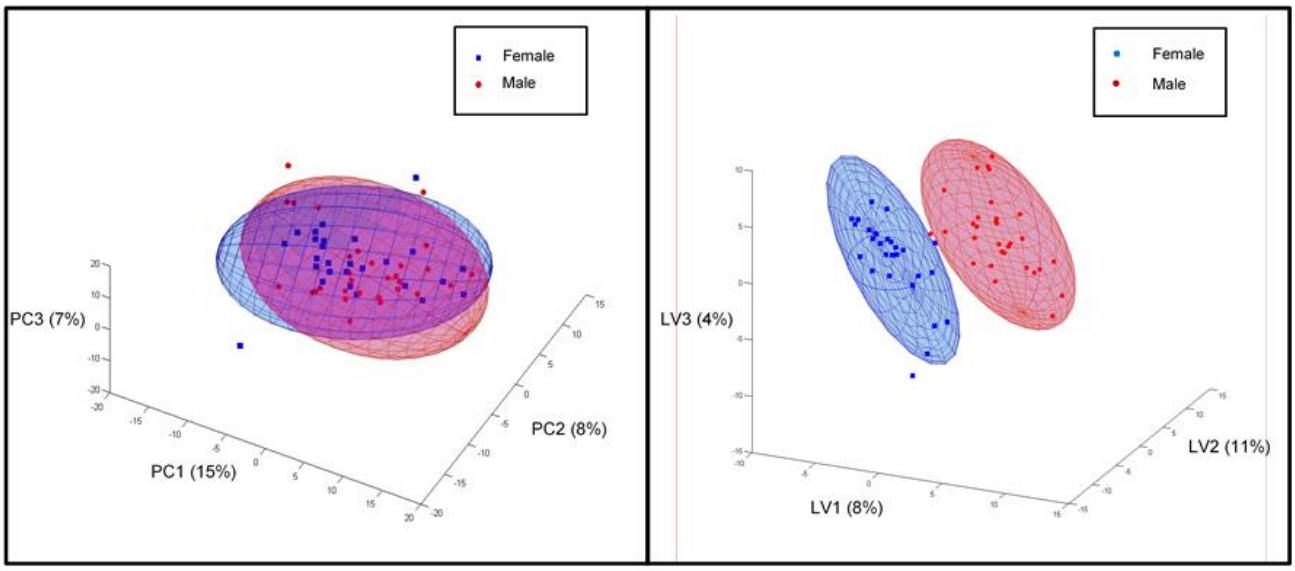


Table 4-7. Partial least squares analysis comparing the top VIP scores of urine organic acids in female and male cheetahs

Metabolites	*Female mean (mmol/L) (N = 25)*	*Male mean (mmol/L) (n = 32)*	*PLS VIP scores*	*Effect Size (r-value)*	*Mann-Whitney U (p-value)*	*Metabolic origin*
N-Acetylphenylalanine	0.593	0.292	5.061	0.376	0.004	Enteric bacteria/Phenylalanine
2-Hydroxy-5-methoxybenzoic acid	0.090	0.022	4.456	0.366	0.005	Unknown
N-Hexanoylglycine	13.525	7.336	4.140	0.338	0.010	Fatty acid metabolism
N-Tiglylglycine	0.739	0.351	4.123	0.303	0.021	Isoleucine metabolism
2,3-Dihydroxybenzoic acid	0.015	0.062	3.674	0.425	0.001	Enteric bacteria
Furoylglycine	0.065	0.013	3.459	0.316	0.016	Carbohydrate metabolism
1,2-Dihydroxycyclohexene	4.607	13.429	3.453	0.321	0.014	Unknown

4.4 Discussion

Urinary organic acids are the intermediate and end-products of several important metabolic pathways. Modern metabolomic techniques allow the quantitative analysis of these metabolites with the potential of providing new information on the physiological and pathophysiological status of these pathways (156). The large number of organic acids present in urine and the complexity of their metabolic origins make their evaluation a daunting task. Nevertheless, the potential insight gained from a systematic study of the organic acid profiles in the cheetah may provide the initial insights necessary to eventually fully understand the diseases that plague them in captive facilities around the world.

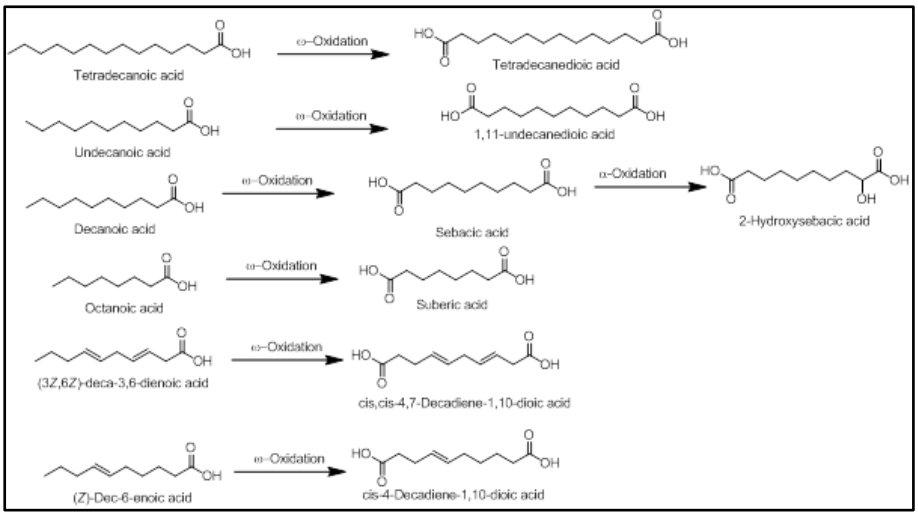
Collection of urine from adult cheetahs can be rather challenging and in most cases requires some degree of sedation or anaesthesia. The urine however provides a fluid, rich in metabolites that is largely unaffected by the stress and physiological changes associated with immobilization. This advantage is, however, only valid if the urine is collected shortly after immobilization. Samples collected from free-ranging cheetahs that were captured in cage traps had to be excluded from this study as there was little consistency in the time they spent in the cage traps prior to immobilization and acute stress could have a dramatic effect on the excreted urinary organic acids. For this reason we decided to only include the two free-ranging cheetahs from AfriCat as both these animals were habituated to the presence of humans and could be darted in a similar fashion to the captive animals. Both animals had also not eaten anything for at least 24 hours prior to immobilization. It is therefore interesting that the PLS-DA and PCA clearly clustered these two individuals closely together and separated them clearly from the captive cheetahs. The unsupervised PCA clearly places the urine organic acid profiles of these two free-ranging cheetahs as clustering outliers, indicating that the variability between these two individuals in minimal compared to the differences between them and the captive cheetahs.

The dicarboxylic acids derived from the oxidation of several saturated and unsaturated medium-chain fatty acids were found at higher concentrations in the free-ranging cheetahs than in the captive cheetahs. Omega oxidation catalysed by CYP4A and CYP4F sub-families is a detoxification reaction for accumulating fatty acids in the cytoplasm. The urinary excretion of dicarboxylic acids has been shown to increase through ω-oxidation when mitochondrial β-oxidation is inhibited or in certain inborn errors of mitochondrial or peroxisomal β-oxidation (171). Similar dicarboxylic acids have however also been detected in the urine of rats fed a ketogenic (high fat/low carbohydrate) diet (172). In extreme situations the dicarboxylic acids may enter the mitochondria again and conjugated with glycine by glycine-N-acylase. In patients with medium-chain acyl–CoA dehydrogenase deficiency (MCAD), dicarboxylic acids

(like adipic acid, suberic acid and sebacic acid) accumulate in mitochondria where they are detoxified by conjugation with glycine, producing several acylglycine conjugates (N-adipylglycine, N-suberylglycine and N-sebacylglycine) which are detected at high concentration in their urine (22). Such dicarboxylic-acylglycine conjugates were, however, not detected in the cheetah urine. It therefore seems likely that the fatty acids accumulate in the cytoplasm in the cheetahs but not to such an extent that accumulation also occurs inside the mitochondria. The fatty acids and their corresponding dicarboxylic acids detected in the cheetah urine are illustrated in Figure 4-5.

Cheetahs in captivity are often fed the fairly lean muscle meat of eviscerated domestic farm animals (donkeys, horse, cattle or chickens). The intra-abdominal fat is often discarded together with the internal organs. In the wild, cheetahs are known to frequently open up the abdomen of their prey, consuming the abdominal organs and visceral fat before moving on to the rest of the carcass (59,61). It is therefore likely that captive cheetahs receive less fat in their diets than their free-living counterparts resulting in lower dicarboxylic acid concentrations in their urine. Fatty acid concentration could also potentially increase during periods of prolonged fasting due to increased fatty acid mobilization. The male free-ranging cheetah had a low BMI of 47.47 (male mean = 55.35), while the female had a BMI of 50.86 (female mean = 50.22). Despite this variation in BMI, both animals had similar urine organic acid profiles. Even though it is possible that these free-ranging individuals had less frequent access to food, a study in rats showed that, although dicarboxylic acids formed from the ω-oxidation of fatty acids are produced at a higher rate during starvation, they are quickly consumed via the Krebs cycle and thus excreted at lower concentrations in the urine (173). An increased dietary fat intake therefore seems to be a more plausible explanation for the increase in fatty acid metabolite concentrations in the urine of the free-ranging cheetahs.

Figure 4-5. ω-oxidation of various saturated and unsaturated medium-chain fatty acids and the corresponding dicarboxylic acids detected in cheetah urine.



A compound tentatively identified as N^1,N^5-dimethylpentane-1,5-diamine and a still-unidentified compound (Unknown 362), were detected at respective mean concentrations of over 300 and 3000-fold higher in captive cheetahs when compared to the two free-ranging cheetahs. The mass spectrum and fragmentation of N^1,N^5-dimethylpentane-1,5-diamine is shown in Figure 4-6. This compound has to our knowledge, not been detected in the urine of any species before. It is likely to be metabolised from cadaverine through transmethylation, as shown in Figure 4-7, in a similar way to the production of substances such as N,N-dimethyltryptamine (DMT) (174). Cadaverine is a malodourous diamine produced by the bacterial decarboxylation of lysine (175) and has been shown to be mildly toxic to rats (176). In captive cheetahs, the high protein intake from muscle meat, could potentially result in increased lysine being available for colonic bacterial decarboxylation. The potential toxic effects of cadaverine or its methylated derivative are unknown in the cheetah. The high concentrations, detected in the urine of captive individuals, warrants further investigation of the potential toxicological effects of these compounds.

Figure 4-6. Mass spectrum and fragmentation of a compound detected at high concentration in the urine of captive cheetahs, tentatively identified as N^1,N^5-dimethylpentane-1,5-diamine

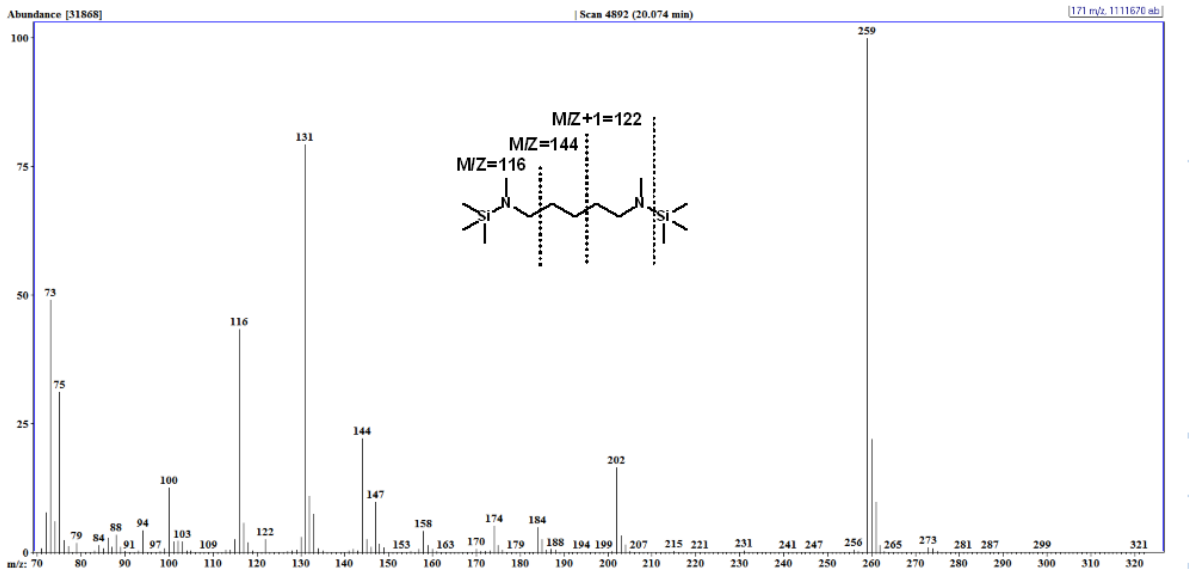
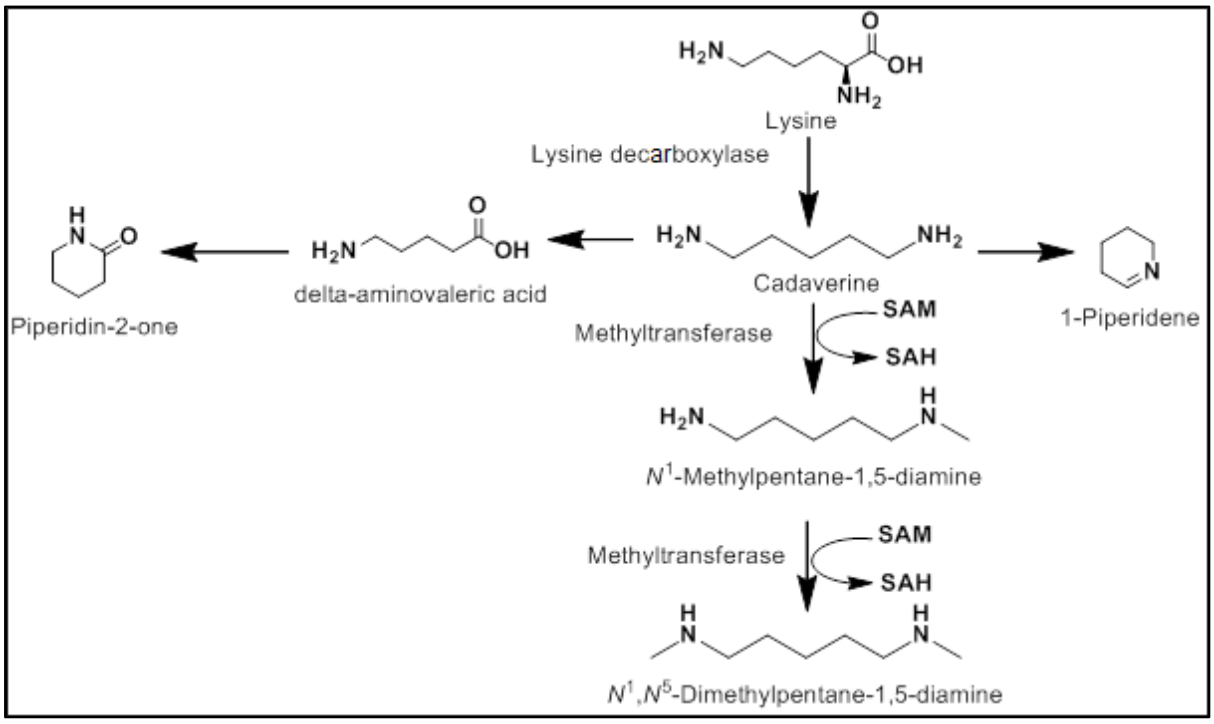


Figure 4-7. Proposed metabolic pathway for the metabolism of cadaverine and the formation of N^1,N^5-Dimethylpentane-1,5-diamine. SAM = S-Adenosyl-L-methionine, SAH = S−Adenosyl homocysteine.



Phenolic compounds associated with the intestinal microbial fermentation of phenylalanine (4-hydrocinnamic acid) and tyrosine (4-hydroxyphenylacetic acid, 4-hydroxymandelic acid, 2-hydroxyphenylpropionic acid, and 4-hydroxyphenyllactic acid) as well as their corresponding glycine conjugates (N-phenylacetylglycine, hippuric acid, N-phenylpropionylglycine, N-4-hydroxyphenylacetylglycine and N-cinnamoylglycine) were also detected at high concentration in the cheetah urine. The known metabolic pathways for the formation of these compounds are illustrated in Figures 4-8 and 4-9.

Figure 4-8. Known pathways for the metabolism of phenylalanine. Bacterial metabolism shown by dotted arrows, while solid arrows indicate mammalian biosynthesis.

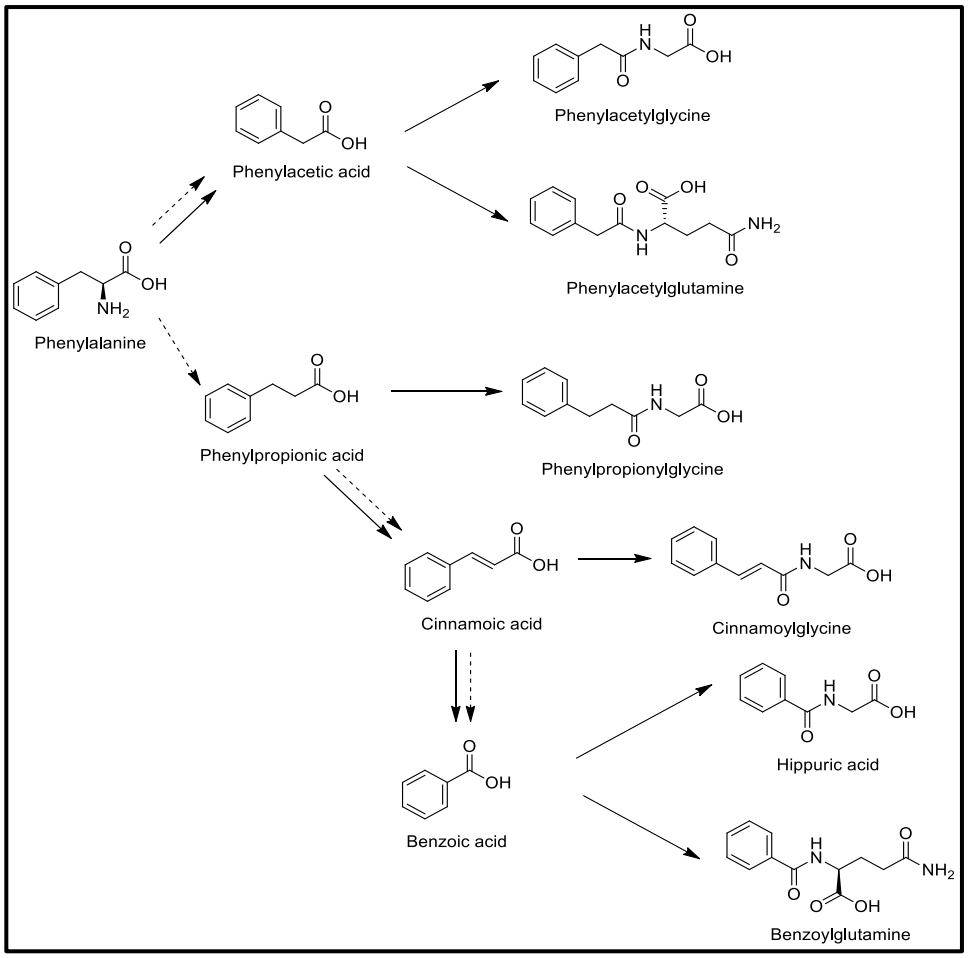
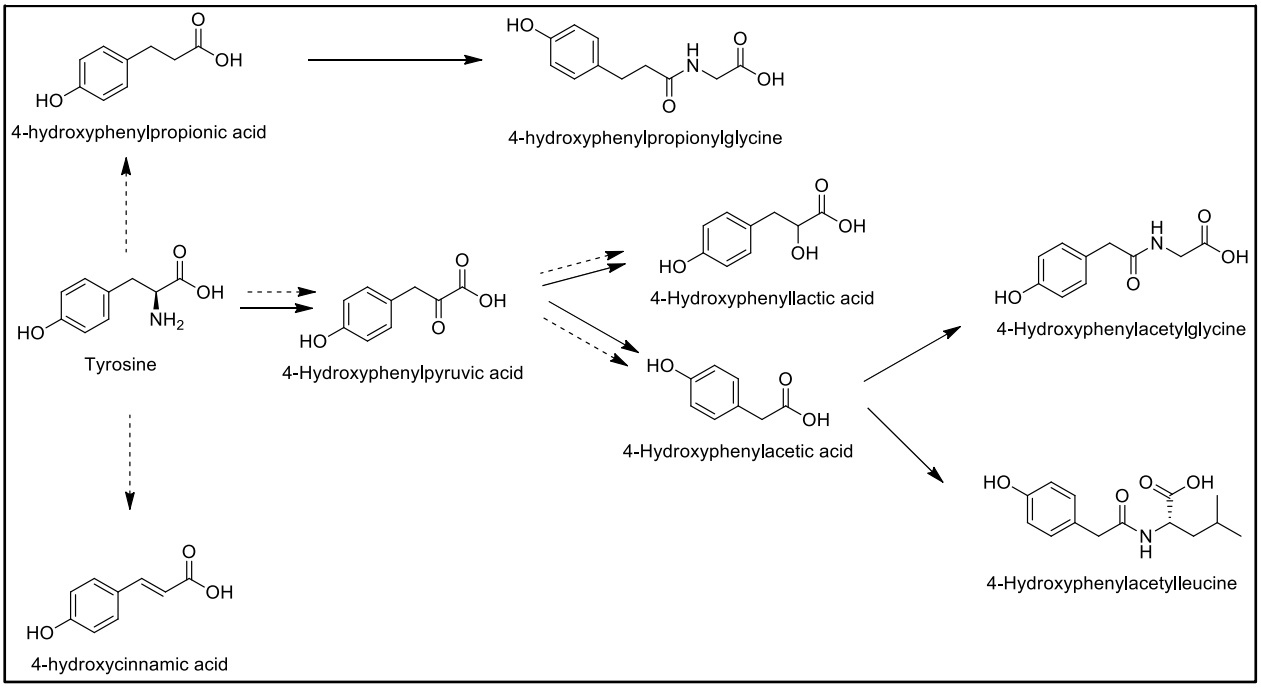


Figure 4-9. Known pathways for the metabolism of tyrosine. Bacterial metabolism shown by dotted arrows, while solid arrows indicate mammalian biosynthesis.



Microbial fermentation of the aromatic amino acids has been shown in humans and laboratory rodents to occur primarily in the distal colon and results in the formation of a range of aromatic phenolic compounds, often referred to as xenobiotics, which are absorbed by the host and then modified by endogenous metabolism or excreted unchanged in the urine (177,178). Amino acid conjugation in the mammalian hepatic and renal cells appears to be the primary method of metabolising these compounds prior to urinary excretion. This reaction takes place within the mitochondria in a two-step process which firstly involves the formation a xenobiotic acyl-coenzyme A (acyl-CoA) thioester followed by conjugation to glycine (179). There is mounting evidence that many of the microbial phenolic compounds absorbed from the colon as well as the reactive acyl-CoA thioesters formed prior to conjugation are potentially toxic to the host organism (reviewed in (180)). For example, it has been demonstrated that, even at fairly low concentrations, the phenolic acids formed by bacteria during sepsis stimulate the production of reactive oxygen species (ROS) in isolated liver mitochondria and inhibit the activity of complex 1 in the electron transport chain (181). The acyl-CoA thioesters are reactive and able to modify proteins, inhibit enzymes and act as alternative substrates leading to the production of abnormal metabolites (179). Shen reported that tyrosine metabolites including 3,4-dihydroxyphenylalanine, tyramine, 4-hydroxyphenylpyruvic acid, 4hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid are potent non-competitive inhibitors of the enzyme dihydropteridine reductase (DHPR) (182). DHPR is required for the enzymatic regeneration of tetrahydrobiopterin (BH4), an essential cofactor for enzymes of key metabolic importance

including four aromatic amino hydroxylases, alkylglycerol mono-oxygenase and three nitric oxide synthase isoenzymes (183). Although BH4 is produced *de novo* from guanosine-5-triphosphate, genetic mutations that result in DHPR deficiency lead to significant reductions in BH4 availability. In humans, this rare inherited disorder manifests in severe neurological disease associated with catecholamine and serotonin deficiencies with or without hyperphenylalaninaemia (184). BH4 is almost ubiquitous in mammalian cells and plays an important role in monoamine neurotransmitter production, vascular dysfunction, immune function and possibly pain sensitivity (183). Despite the demonstrated inhibition of DHPR by tyrosine metabolites in human and sheep liver by Shen, acquired BH4 deficiencies due to DHPR inhibition have to our knowledge not been reported in the literature. The reduced function of the aromatic amino acid hydroxylases due to BH4 oxidation leads to depletion of the monoamine neurotransmitters as well as reduced urinary excretion of their end-stage degradation metabolites, namely 5-indoleacetic acid (5-IAA), vanillylmandelic acid (VMA), homovanillic acid (HVA) and 3-methoxy-4-hydroxyphenylglycol (MHPG). Figure 4-10 shows the simplified pathway of monoamine neurotransmitter synthesis and degradation.

The scatterplots shown in Figure 4-11 indicate the relationships between various phenolic metabolites, VMA and HVA excreted in the cheetah urine. In most cases the plots show a nonlinear inverse correlation, of varying degree, between the phenolic compounds and the VMA/HVA concentrations. Higher urinary concentrations of VMA and HVA (>2mmol/L) were almost always associated with very low phenolic compound concentrations. These relationships support the suggested DHPR inhibition by some aromatic amino acid metabolites and subsequent reduced aromatic amino hydroxylase activity in several of these cheetahs. . Furthermore, the captive cheetahs excreted higher mean concentrations of 4-hydrocynnamic acid, 4-hydroxycinnamc acid 4-hydroxyphenylacetic acid, 4-hydroxyhippuric acid, 4-Hydroxyphenylpyruvic acid, N-phenylpropionylglycine, N-4-hydroxyphenylacetylglycine, N-4-hydroxyphenylpropionylglycine and N-phenylacetylglycine (Figure 4-12). Hippuric acid, the glycine conjugate of benzoic acid, was the only phenolic metabolite excreted at higher mean concentrations in the free-ranging cheetahs (Figure 4-13). Free-ranging cheetahs also excreted more VMA and HVA than their captive counterparts (Figure 4-14). Taken together, these findings suggest that several of the aromatic amino acid metabolites produced by enteric microbial fermentation may have a negative effect on the production of the monoamine neurotransmitters in a number of captive cheetahs.

Figure 4-10. Diagrammatic representation of the synthesis and degradation of monoamine neurotransmitters from aromatic amino acids. Tetrahydrobiopterin (BH4) acts a cofactor for phenylalanine hydroxylase (PH), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TRH). Dihydropteridine reductase (DHPR) is required for the regeneration of BH4 from q-dihydrobiopterin (q-BH2). Serotonin, dopamine, noradrenaline and adrenaline are oxidised to 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid, 3-methoxy-4-hydroxyphenylglycol (MHPG) and vanillylmandelic acid respectively.

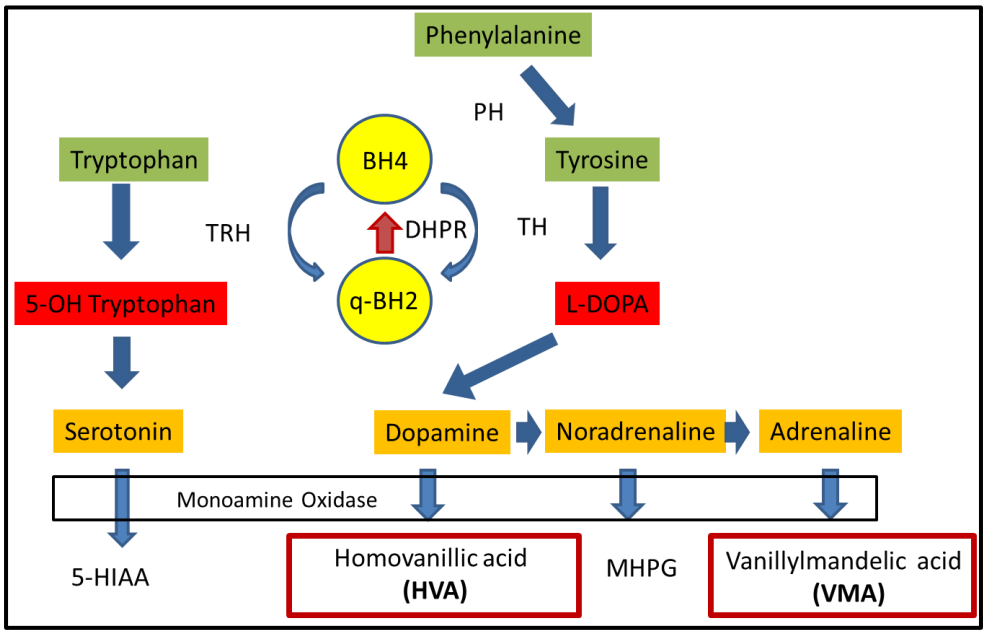


Figure 4-11. Scatterplots showing the relationship between selected phenolic compounds, Vanillylmandelic acid (VMA) and Homovanillic acid (HVA) in cheetah urine.

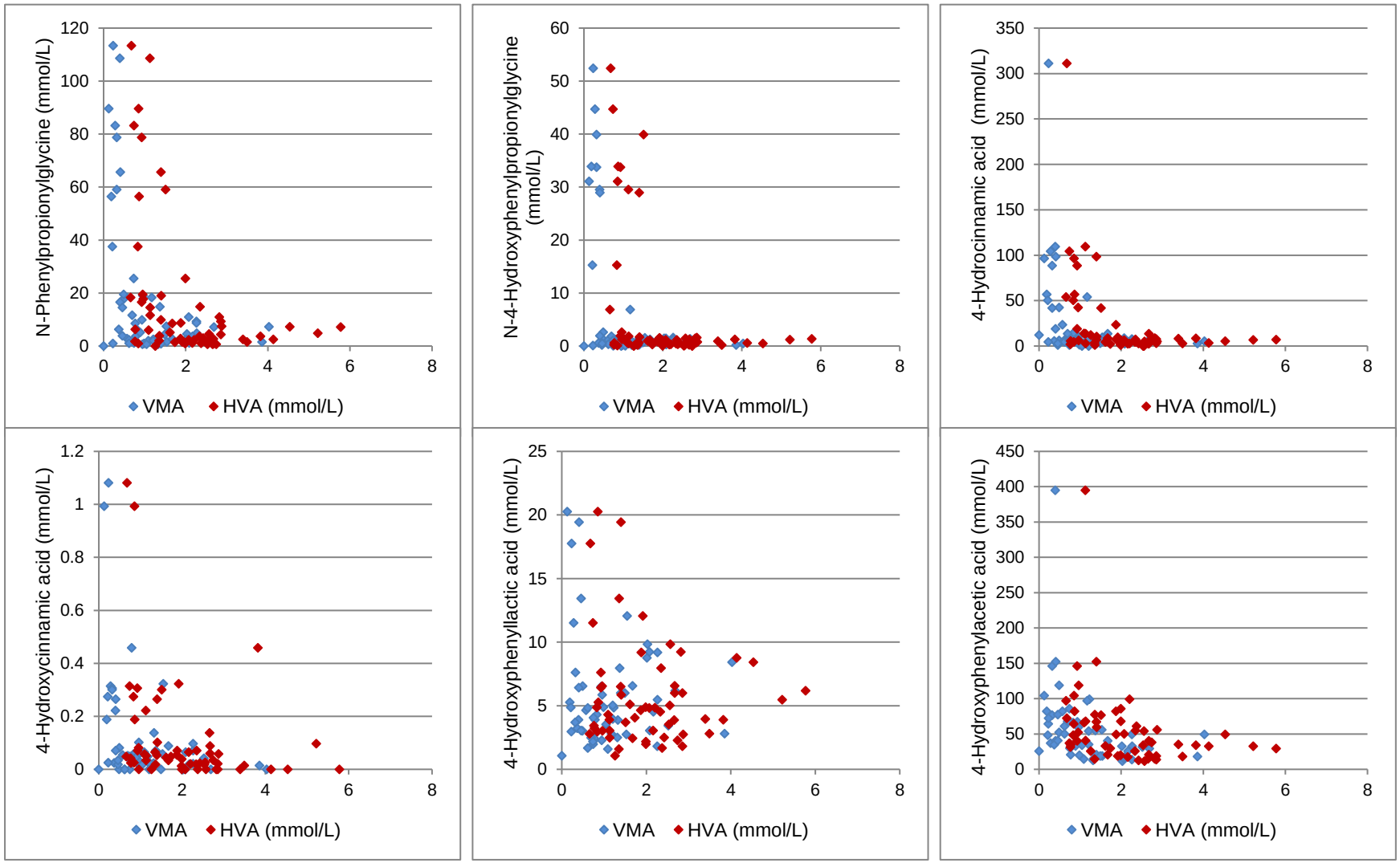


Figure 4-12. Selected mean urine phenolic metabolite concentrations with standard error bars in free-ranging (n=2) and captive (n=55) cheetahs

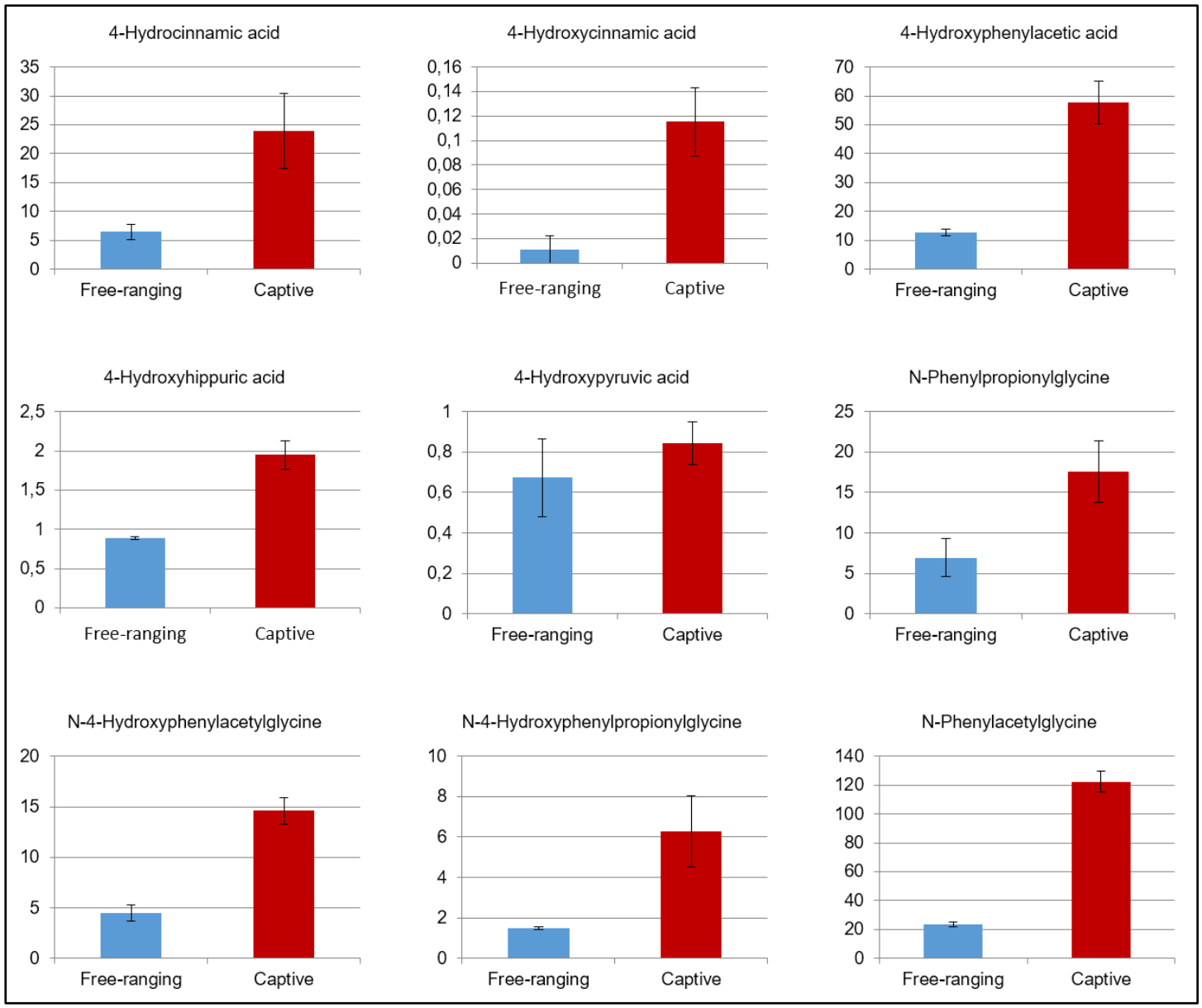


Figure 4-13. Mean urine benzoic acid and hippuric acid concentrations with standard error bars in captive and free-ranging cheetahs.

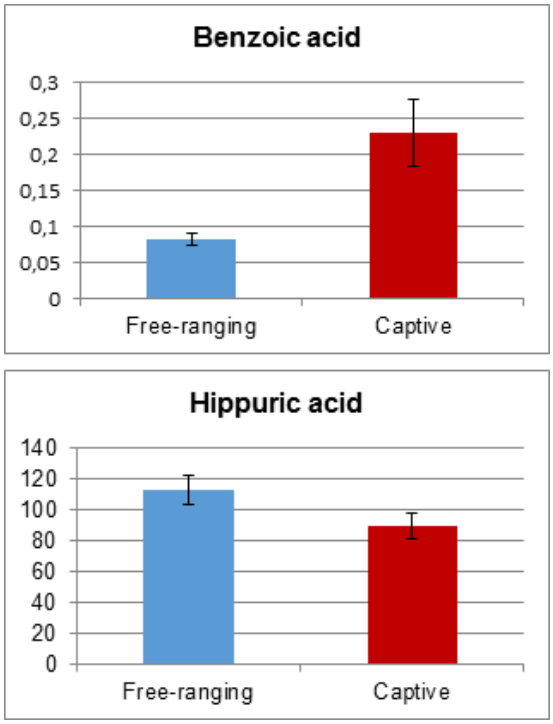
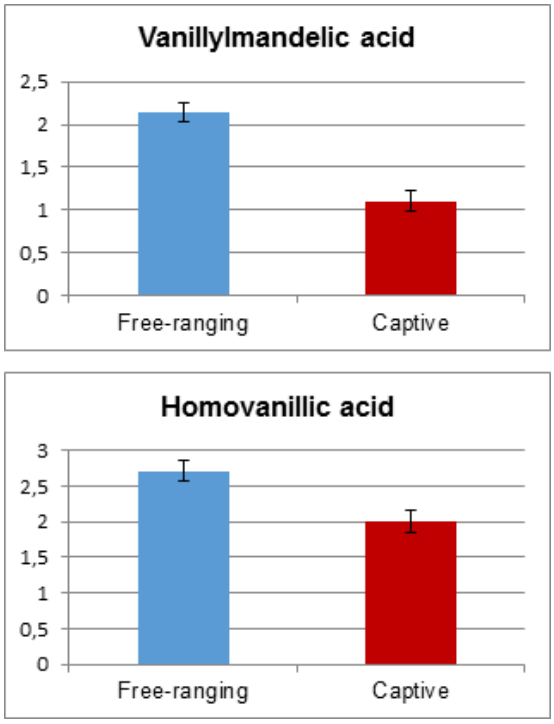


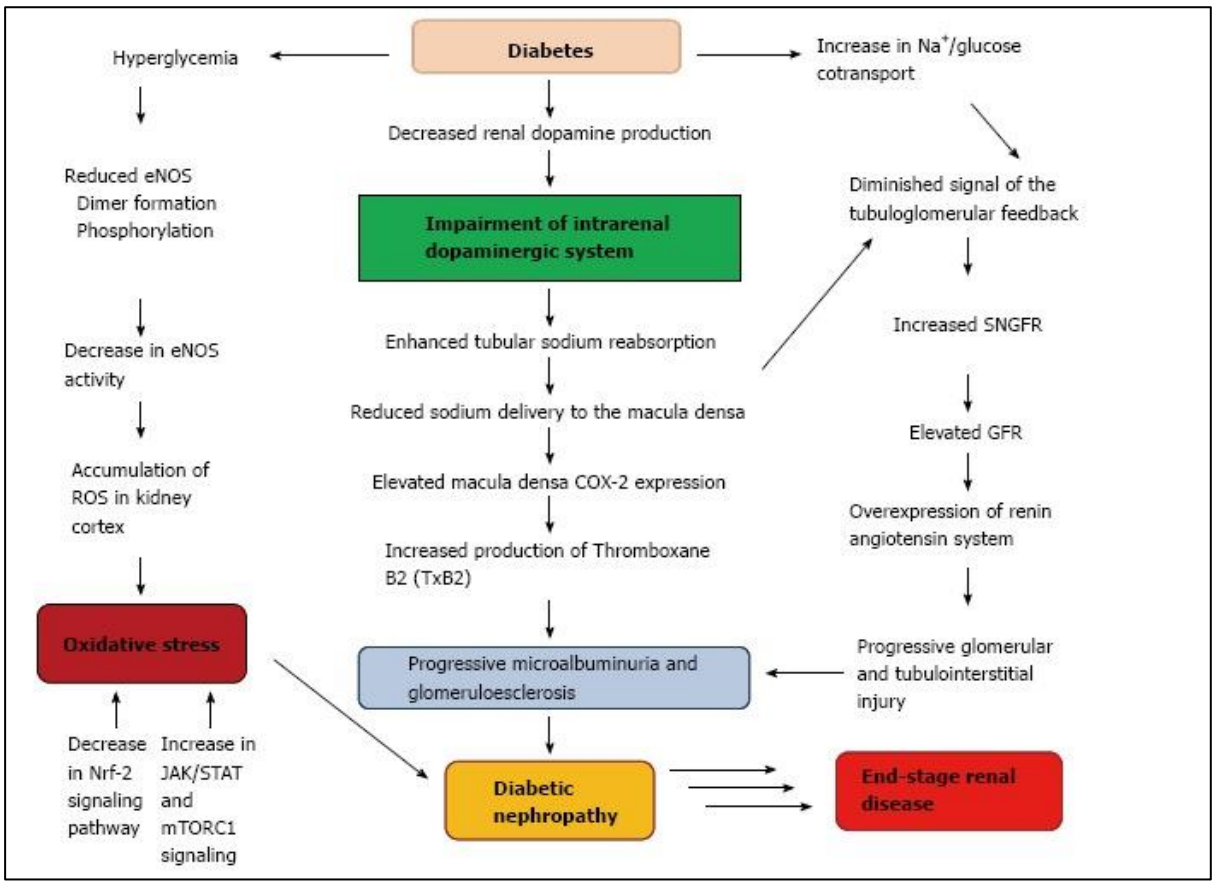
Figure 4-14. Mean urine concentrations with standard error bars of vanillylmandelic acid and homovanillic acid in captive and free-ranging cheetahs.



Both dopamine and serotonin play important roles as neurotransmitters, but also have important functions within the cardiovascular, renal and gastrointestinal systems. Renal dopamine increases glomerular filtration rate and causes natriuresis by inhibiting tubular sodium reabsorption and increasing the production of natriuretic prostaglandin E_2 (185). Dopamine also appears to be an important modulator in the pathogenesis of gastrointestinal ulceration. Dopamine and its agonists have a protective role in stress-induced gastric ulceration; improve duodenal motility; influence gastric, duodenal and pancreatic secretions, and increase blood flow to the stomach, duodenum, pancreas and colon (186). Central nervous system depletion of serotonin, dopamine and noradrenaline have been implicated in the pathogenesis of major depressive disorder (MDD), a condition in which hyperactivation of the hypothalamus-pituitary-adrenal (HPA) axis is also a common feature. Evidence suggests that the serotonergic and HPA neuroendocrine systems are linked. However, the interactions between these systems are complex and individually variable (187). Nevertheless, these endocrine system interactions do raise interesting questions about the possible dysregulation of the monoamine and HPA systems in cheetahs, where adrenal hyperplasia and excessive cortisol production commonly occur in captive individuals (16).

Renal glomerulosclerosis is a very common finding at necropsy in captive cheetahs, characterised by thickening of the glomerular and peritubular basement membranes as well as cortical and tubular atrophy, cortical fibrosis and lympho-plasmacytic infiltration (9). These glomerular lesions resemble those of diabetic nephropathy in humans, yet cheetahs very rarely develop persistent hyperglycaemia or diabetes mellitus. In a mouse study, selective inhibition of intrarenal dopamine accelerated the development of diabetic nephropathy (188). The relationship between inhibition of the dopaminergic system and the development of diabetic nephropathy are illustrated in a diagram by Choi *et al* (189) (Figure 4-15). It is therefore possible that, in the absence of diabetes mellitus, reduced renal dopamine production, due to suppression of the aromatic amino acid hydroxylases, may play a role in the pathophysiology of glomerulosclerosis in cheetahs.

Figure 4-15. Diagram shown in Choi et al (189) illustrating the association between diabetes and the renal dopaminergic system in the pathophysiology of diabetic nephropathy.



Glycine is the simplest of the 20 amino acids found in animal protein and is not generally considered to be an essential amino acid, since it can be synthesized from several substrates. Besides being an important protein component, glycine also plays an important role in neurotransmission and is required for the production of glutathione, creatine, porphyrins and purine nucleotides. In this study cheetahs were shown to excrete glycine conjugates in large concentrations in their urine. This, together with their apparent large requirements of creatine, would necessitate substantial amounts of glycine to be ingested or produced to meet their metabolic demands. The glycine content of muscle meat is 3 to 4 times lower than in collagen-rich tissues and organ meats (190). Cheetahs in captivity that are largely fed muscle meat are therefore at potential risk of becoming glycine deficient. In their review, Badenhorst *et al* demonstrated how the glycine conjugation of an ingested phenolic compound, namely benzoic acid, could lead to a glycine deficiency in humans (180). They go on to argue that, in the absence of sufficient glycine, reactive acyl-CoA thioesters accumulate in the liver and renal mitochondria, resulting in the sequestration of coenzyme A (CoA). Since CoA plays a pivotal role in a wide range of metabolic processes, a shortage of this molecule due to sequestration could therefore have serious and far reaching metabolic consequences (191). Beside the depletion of CoA, the acyl-

CoA thioesters have been shown to have several potential toxic effects (191). Both glycine and CoA reserves have been shown to limit the rate of benzoic acid conjugation in rats (192). With a reduced rate of glycine conjugation benzoic acid would accumulate and the relative production of hippuric acid would be reduced, altering both the serum and urine benzoic acid to hippuric acid ratios. On average, the captive cheetahs excreted almost three times as much unconjugated benzoic acid and about 20% less hippuric acid than the free-ranging cheetahs (Figure 4-13). Since CoA synthesis requires pantothenic acid, a deficient intake of this B vitamin could also contribute to a reduction in the conjugation rate of phenolic compounds. The urinary excretion of pantothenic acid did not, however, differ between the captive and free-ranging cheetahs. These findings suggest that, in some captive cheetahs, glycine and/or CoA depletion may result from both a reduced glycine intake and an overwhelming metabolic demand for these compounds for the conjugation of large concentrations of microbial phenolic metabolites.

Since the incidence and severity of glomerulosclerosis, adrenal hyperplasia and gastritis correlate positively with age in captive cheetahs (9,193), we evaluated the association between urinary organic acid metabolites and the age of the cheetahs (Table 4-3). The three metabolites that correlated most strongly with age, namely pantothenic acid, citramalic acid and glutaric acid, are all in some way associated with CoA metabolism. Pantothenic acid, an important component of CoA and acyl carrier proteins, correlated negatively with age ($r = -0.408$, $p=0.001$). This B vitamin is normally abundant in many food sources, mostly in the form of CoA, but concentrations in muscle meat are approximately 7 to 10 times lower than in organ meats such as liver and kidney (194). Since dietary intake in captive cheetahs is, however, unlikely to change with age, reduced intestinal absorption or the increased utilization of pantothenic acid could explain the reduced renal excretion of this vitamin in older cheetahs. An increased demand for CoA could reduce the available pool of pantothenic acid and therefore result in reduced renal excretion. Citramalic acid is a degradation product of itaconic acid in a process that also requires CoA (195). The negative correlation with age could thus also be explained by a relative age-related CoA deficiency. Glutaric acid is an intermediate in the degradation pathway of tryptophan, lysine and hydroxylysine. In mammals the utilization of glutaric acid requires its mitochondrial activation to glutaryl-CoA, a reaction that once again necessitates CoA (196). The positive correlation with age in the cheetahs could also be explained by a decline in CoA concentrations with advancing age. The dramatic decline in urinary CR excretion relative to age (as discussed in Chapter 3) is also of interest, since a reduction in glycine could limit the production of its precursor creatine, explaining this decline. These findings thus support the hypothesis that older cheetahs suffer from a progressive glycine and potentially associated CoA deficiency. The actual mechanisms of this age-related decline are however unclear.

Urine organic acids associated with aromatic amino acid metabolism also featured strongly in the correlation between body mass index scores and urinary organic acid concentrations. Phenylacetic acid, 4-hydroxycinnamic acid, hippuric acid and 2-hydroxyhippuric acid correlated positively with BMI in the female cheetahs. This relationship was however not seen in the males. Increasing age in the cheetahs was not associated with any significant decline in BMI and none were considered either over or underweight. The BMI does not differentiate between body fat and lean muscle tissue and therefore provides no indication of body fat percentage. It is thus unclear if the metabolite concentrations that correlate with BMI are associated with changes in muscle or fat mass. The positive association found between these phenolic compounds and BMI in the female cheetahs is in contrast to a study in dogs where the feeding of a restricted diet (25% less food than controls), and presumably lower body weights, resulted in increased excretion of some aromatic amino acid metabolites (hippuric acid, phenylacetylglycine and 4-hydroxyphenylacetic acid) (163). The variation in BMI score in the cheetahs could result from a variety of factors including differences in food intake, feeding competition, intestinal micro-floral composition and a range of potential disease processes. Metabolomic data in cheetahs would have to be evaluated in controlled feeding trials to shed further light on these associations.

The absorption, conjugation and urinary excretion of microbial phenolic metabolites from amino acid precursors have been poorly studied in carnivores, and very little is known about their impact on metabolism and health. Depauw *et al* compared the faecal excretion of short-chain- (SCFA) and branched-chain fatty acids (BCFA) as well as indole and phenol in cheetahs fed either supplemented beef or whole rabbits and found that on the whole-rabbit diet, cheetahs produced less BCFA, putrefactive indole and phenol (197). Although total faecal SCFA concentration remained unaltered, the acetic acid to propionic acid ratio increased and butyric acid decreased on the whole rabbit diet. They concluded that this decrease in putrefactive metabolites could be caused by the higher intake of poorly digestible tissue like skin, bone and collagen, but acknowledged that a higher fat to protein ratio of the whole rabbit diet may also have had an influence on the results. These findings are consistent with our data and support the idea that the high muscle meat content in the diets of captive cheetahs results in the increased fermentation of the aromatic amino acids by enteric microbes and the production of potentially toxic phenolic compounds.

4.5 Conclusion

The urinary organic acid profiles evaluated in these cheetahs provide new insights into potential disruption of the monoamine and related neuroendocrine systems. Furthermore, the required detoxification of these phenolic compounds, through glycine conjugation, could result in the chronic depletion of both glycine and sequestration of CoA, with the associated negative metabolic consequences. Further studies can now be designed to test these hypotheses and to

correlate these changes with the incidence and severity of the diseases suffered by cheetahs in captivity. The small sample size from free-ranging cheetahs is a severe limitation in this study and future sampling efforts will need to focus on these animals to further test these hypotheses. This study however demonstrates the value of an untargeted metabolomic approach in the generation of new hypotheses which will no doubt advance future disease research in this species.

CHAPTER 5
SERUM AND URINE AMINO ACIDS

5.1 Introduction

Amino acids are not only important components of structural proteins but also important metabolites, either in their free form or in peptides, playing a wide role in neurotransmission, detoxification, regulation of pain and inflammation, cholesterol metabolism and acid-base physiology (198). Besides the 20 amino acids incorporated in proteins, a large number of other compounds, with an amine group, are also considered amino acids in the body. Some of the amino acids used for protein synthesis are modified during proteolysis, while others are formed during specific metabolic processes or consumed in the diet (199)

The protein requirements of adult domestic cats has been shown to be 2-3 times higher than that of non-carnivores (200). This increased protein requirement could either be due to a higher requirement for one or more of the essential amino acids or due to a higher demand for nitrogen. In adult cats the demand for dietary protein appears to be necessary for both reasons. Kittens, other than having a higher requirement for arginine, have similar essential amino acid needs to other growing mammals (201). When omnivores are fed a low-protein diet, they conserve amino acids by reducing the activity of aminotransferases and other enzymes involved in protein catabolism. Cats, on the other hand, show limited adaptation in the activity of these enzymes in response to the level of dietary protein and continue to use protein for energy even when fed a low-protein diet (75). Cats have higher requirements for dietary taurine, arginine, methionine and cysteine, presumably because these amino acids are abundant in their natural diet. The endogenous synthesis of these metabolites would therefore be unnecessary and energy inefficient. Even when faced with dietary deficiencies of these amino acids, their utilization in cats remains high (202).

The free serum amino acid pool is derived primarily from; the digestion of endogenous proteins and peptides, absorbed into the circulation after being sloughed or secreted from the gastro-intestinal tract; dietary protein digested and absorbed from the gastro-intestinal tract and intracellular protein turnover or degradation (199). Serum concentrations of amino acids appear to be tightly controlled by homeostatic mechanisms and remain fairly constant after initial postprandial fluctuations in healthy adult humans (203), but have been shown to vary significantly during growth, disease and various catabolic conditions (204-209). Serum or plasma amino acids are influenced by dietary protein and amino acid intake (210,211). Unlike other macronutrients, amino acids cannot be stored when provided in excess. Dietary amino acids can be synthesised into proteins, but these are only produced in quantities necessary for specific functions. Surplus

amino acids therefore have to be metabolised and this has important implications in carnivores, where a significant proportion of the diet is made up of protein.

The pattern of urinary excretion of amino acids varies tremendously between different mammalian species (212,213), but tends to be more uniform within a species (214). Urinary amino acid concentrations are frequently evaluated in suspected cases of inborn-errors of metabolism, where dramatic aminoacidurias are apparent. The excretion patterns are however more subtly affected by diet and age, while modified patterns of excretion have been documented in various disease states (214).

Serum or plasma amino acid profiles have been reported in domestic kittens fed purified diets (201,215) and in adult cats fed commercial diets (216). A single metabolomic study that included plasma amino acid quantification in domestic cats, only recently appeared in the literature (217). Only limited serum and urinary amino acid profiles of non-domestic felid species have, to our knowledge, been reported (212,218). A number of studies have focused on the excretion of felinine and isovalthine, two sulphur-containing amino acid excreted in the urine of a few felid species (219). The biological significance of these sulphur amino acids remains unclear, but it is speculated that they may act as precursors to certain pheromones (220).

In order to develop a better understanding of the unique metabolic processes and nutritional requirements of these animals we determined the serum and urine amino acid profiles of captive cheetahs in an untargeted fashion using gas chromatography mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (LC-MS-MS).

5.2 Materials and methods

5.2.1 Samples

Captive cheetahs at the AfriCat Foundation in Namibia were immobilized in June/July 2013 as described in Chapter 2 after being starved for 24 to 48 hours. Blood samples were collected within 15 minutes of immobilization from 42 adult cheetahs (Table 5-2), while urine samples were available within the same time frame from 26 of these individuals (Table 5-4).

The blood samples were collected aseptically from a jugular vein with a 20ml syringe and 18 gauge needle. The blood was transferred into 6ml BD Vacutainer® tubes (Becton, Dickinson and Company, South Africa) and allowed to clot for 40 minutes on ice. Samples were then centrifuged at 3 500 rpm for five minutes, the serum was pipetted off into 1.8 ml Cryovials® (Thermo Scientific, South Africa) and immediately frozen at -20 °C.

Urine was collected by direct urethral catheterization using a 6 FG dog urinary catheter into a 20ml syringe. The urine was placed into 1.8 ml Cryovials® (Thermo Scientific, South Africa, 1401) and immediately frozen at -20 °C.

The samples were transported to the laboratory on dry ice and then kept frozen at -80°C until analysis.

5.2.2 Urine creatinine, specific gravity and fractional excretion

In the laboratory, urine was thawed and allowed to warm to room temperature. The urine creatinine concentrations were determined by an enzymatic method on an Indiko Clinical Chemistry Analyzer (Thermo Scientific, South Africa) using the manufacturer's kit and instructions. The enzymatic reaction involves the conversion of creatinine to sarcosine with the aid of creatininase and creatinase. Sarcosine is then converted to glycine, formaldehyde and hydrogen peroxide by sarcosine oxidase. The hydrogen peroxide reacts with 4-aminophenazone and HTIB to form quinone imine chromogen in a reaction catylysed by peroxidase. The colour intensity is directly proportional to the concentration of creatinine. The urine specific gravity (SG) was measured with a hand held refractometer (RHC-200), calibrated with distilled water. The creatinine concentrations of 20 of the serum samples were determine at the Onderstepoort Veterinary Academic Hospital on a Cobas Integra 400 Plus Analyser® (Roche, South Africa).

The fractional excretion of amino acids provides an indication of the degree of urinary excretion versus reabsorption relative to creatinine and in this study was calculated using the standard equation:

$$Fractional\ amino\ acid\ excretion = 100\ x\ \frac{Urinary\ amino\ acid\ [\ ]\ x\ Serum\ creatinine\ [\ ]}{Urinary\ creatinine\ [\ ]\ x\ Serum\ amino\ acid\ [\ ]}$$

5.2.3 Reagents

The EZ:faast ™ amino acid analysis kits were purchased from Phenomenex Inc. (Torrence, CA, USA). Additional reagents and amino acid standards (DLAla, LAla, LaIle, DaIle, LLeu, DLeu, LVal, DVal, DLNva, LIso, and DLIle), were purchased from Sigma-Aldrich (St Louis, MO, USA) and stable isotope amino acid mixtures (Metabolomics amino acid mix standard) were obtained from Cambridge Isotope Laboratories Inc (Tewksbury, MA, USA).

5.2.4 Amino acid analysis using GC-MS

The gas chromatography – mass spectrometry analysis of the serum and urine amino acids was conducted on a Hewlett Packard HP6890 series GC system with an Agilent 5973N Mass selective detector fitted with an EI ion source, an Agilent Technologies 7683 autosampler and a 7683B injector. The GC system was fitted with a Phenomenex GC FocusLiner liner for HP, split/splitless, w/wool, single taper, 4 mm ID x 78.5 mm L x 6.3 mm OD (part number AG0 4680), and a Phenomenex Zebron EZ AAA amino acid GC column, 10 m x 0.25 mm x 0.25 µm (part number CG0 7169), and an Agilent Technologies gas clean filter system (part number CP17973).

The serum and urine amino acid analyses are based on the methods prescribed in the EZ:faast ™ kit, with the following modifications. 1) Stable isotope amino acid standards (100 µl per sample) were added to ensure accurate quantification of the amino acids. 2) NaOH or HCl is added to the sample to adjust the pH to between 1.5 and 6. 3) The final step (drying of the samples and reconstitution with reagent 6) was omitted.

Helium was used as a carrier gas at a constant flow rate of 1.3 mL/s. Two microliters of the extracts was injected in “splitless” mode with a viscosity delay of 1 second and the injector set to 250 ºC. The oven was set to an initial temperature of 60 ºC for 1 min, and then increased to 110 ºC at 50 ºC/min, immediately followed by an increase to 185 ºC at 20 ºC/min, and then to 235 ºC at 25 ºC/min, and finally to 320 ºC at 30 ºC/min for 1 min.

The MSD transfer line heater was set to 280 ºC, the MS source to 230 ºC, and the MS quadrupole to 150 ºC. Solvent delay was 3.00 min. EMV was set to a gain factor of 1.00. The MSD was set to scan from 40 – 500 m/z with a threshold of 150.

Standard range analysis, using the MSD ChemStation E02.00 software with a linear regression curve fit was used to calibrate the identification and quantification of the amino acids. The results were exported to Microsoft Excel® for further analysis. Urine amino acid concentrations were corrected using urine specific gravity (SG) as described in chapter 3.

5.2.5 Arginine and Citrulline quantification using LC-MS/MS

Serum and urine arginine and citrulline were quantified using LC-MS/MS rather than GC-MS due to their thermal instability. The stable isotope mixture (410 µl) was pipetted into micro-centrifuge tubes to which 10 µl of serum or urine was added. The mixture was centrifuged at 13 000g for 20 minutes after which the supernatant was pipetted into a clean micro-centrifuge tube. The tubes were then placed in a nitrogen dryer at 65°C for approximately 45 minutes. Once dry, 200 µl 3N

buthanolic HCl was added and the tubes were then capped and placed in an incubator, set at 65°C for 15 minutes. The tubes were then placed in a nitrogen dryer for approximately 20 minutes and once dry, 100 µl of the mobile phase (acetonitrile:water [50:50] with 1% acetic acid) was added. The samples were then transferred to GC vials for analysis.

Citrulline and arginine was analysed on a Agilent 6420 Triple Quad MS-MS (in the ESI mode, Delta EMV=500) linked to a Agilent 1260 HPLC. Citrulline-d4 and arginine-d4 were used as internal standards. Materials were prepared as described for acylcarnitine analysis with butanol/HCl as derivatization reagent. The butyl esters (5 μl) was injected with direct infusion. Citrulline and arginine analyses were performed as a precursor scan in positive ion mode. Q1 is set to scan a mass m/z 232.2 236.2, 231.2 and 235.2 respectively for citrulline, citrulline-d4, arginine and arginine-d4 while Q3 is set to determine product ion ion of m/z 70.1, 105.2, 70.1 and 74.3 respectively for the amino acids. The settings of the MS was as indicated in Table 5-1. Source parameters: Gas temp-280 C, Gas flow-7.5 l/min, Nebulizer-28 (psi), Capillary-3500 V

Table 5-1. Agilent 6420 Triple Quad MS QQQ MRM settings.

Name	ISTD	Prec Ion	Prod Ion	Dwell	Frac (V)	CE (V)	Cell Acc (V)	Polarity
Citr-d4	Yes	236.2	105.1	45	85	16	7	+
Arg-d4	Yes	235.2	74.3	45	100	40	7	+
Citr	No	232.2	70.1	45	94	28	7	+
Arg	No	231.2	70.1	45	93	28	7	+

5.2.6 Statistical analysis

Variables in which more than 40 % of the data points were zero were eliminated and not considered for analysis. As some cystine is reduced in urine to two cysteine molecules, the total cystine/cysteine was calculated as follows: Total cystine/cysteine = cystine [ ] + (cysteine [ ]/2). Data was tested for normality using the D'Agostino and Pearson omnibus (K2) normality test. Spearman's rho correlation coefficients were used to evaluate the relationship between age, body mass index (BMI) and the various amino acid concentrations. Unpaired t-tests or Mann-Whitney U tests were used for comparisons between amino acid concentrations and other parameters in males and females. All analyses were performed with GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, California, USA). All statistical tests were 2-tailed and significance was defined as $P<0.05$ in all cases.

5.3 Results

Serum samples were evaluated in 42 adult cheetahs (25 males and 17 females), with ages ranging from 3 to 13 years (Table 5-2). The differences in age between the males and females were not significant (p = 0.32), but BMI values were significantly greater in males than in females (p = 0.0003).

Table 5-2. Age and body mass index data for the cheetahs from which serum samples were collected and analysed.

	Males (n=25)				Females (n=17)				
	Max	**Min**	**SD**	**Mean**	**Max**	**Min**	**SD**	**Mean**	**p-value**
Age (years)	12	3	3.0	6.48	13	3	3.3	7.47	0.32
BMI	60.4	47.16	4.05	55.21	56.16	45.09	3.44	50.29	0.0003

A total of 36 serum amino acids were identified and quantified as shown in Table 5-3. Glutamine was detected at the highest mean concentration (1624 µmol/L) followed by alanine (771.9 µmol/L), arginine (669.8 µmol/L) and glycine (478.7 µmol/L). None of the serum amino acid concentrations differed significantly between males and females. Glycine, hydroxyproline, prolylproline, proline and serine serum concentrations declined significantly with age, but the negative correlations were relatively weak as shown in Figure 5-1. Serum proline-hydroxyproline showed a weak but significant positive correlation with BMI in female cheetahs (r = 0.41, p = 0.0073), while valine showed a weak negative correlation with BMI in males (r = -0.25, p = 0.01). Scatterplots of these relationships are shown in Figure 5-2.

Table 5-3. Summary of the serum amino acid concentration data in 42 adult cheetahs, listed in order from highest mean concentration to lowest.

Amino acid	Minimum	Median	Maximum	Mean	Std. deviation
Glutamine	788,3	1492,0	2981,0	1624,0	567,2
Alanine	317,3	634,5	1824,0	771,9	333,1
Arginine	188,2	700,8	1050,0	669,8	216,6
Glycine	214,8	410,0	1194,0	478,7	214,3
Proline	108,4	213,0	759,5	266,4	136,2
Valine	104,4	180,8	422,4	220,1	90,0
Serine	119,1	186,2	419,2	217,3	79,7
Prolylproline	0,0	173,6	688,0	197,1	118,9
Lysine	91,6	158,0	387,7	187,2	74,9
Glutamic-acid	96,7	144,3	337,4	173,8	66,7
Leucine	74,2	138,5	321,0	166,1	66,1
Allo-Isoleucine	70,9	132,3	317,4	160,1	66,3
Histidine	83,1	133,4	304,8	157,3	59,8
Threonine	78,5	127,7	345,4	154,2	67,3
Pyroglutamic-acid	76,7	127,1	251,5	140,7	50,0
Hydroxyproline	9,2	92,7	465,9	131,6	115,5
Tryptophane	69,2	101,0	238,8	124,5	46,5
Ornithine	22,4	70,1	342,5	108,6	84,0
Isoleucine	35,6	68,4	182,1	85,6	36,6
Tyrosine	42,5	73,0	161,1	83,8	32,3
Phenylalanine	30,8	51,9	220,3	66,6	35,3
Methionine	31,0	52,2	146,8	62,5	27,8
Asparagine	29,6	53,5	131,3	61,5	23,1
Citrulline	34,6	39,9	65,2	41,3	6,1
Aspartic-acid	18,1	28,3	73,6	34,6	14,9
Glycine-Proline	0,3	15,7	84,8	23,6	20,8
Cystathionine	5,0	18,9	69,9	23,5	16,2
α-Aminobutyric-acid	9,2	18,0	61,3	21,7	11,5
Proline-hydroxyproline	2,5	6,1	83,3	14,0	20,4
α-Aminoadipic-acid	3,8	6,0	18,4	7,1	2,9
Cystine	1,5	4,3	25,5	5,7	5,0
β-Alanine	0,6	4,1	21,2	5,0	4,3
Hydroxylysine	0,2	1,8	26,8	4,3	6,7
Methyllysine	0,0	2,7	13,5	3,4	2,9
Sarcosine	0,0	1,8	6,1	2,2	1,3
α-Aminopimelic-acid	0,0	0,1	11,3	0,9	2,5

Figure 5-1. Scatterplots and linear regression showing the significant correlations between age and 5 serum amino acids in cheetahs. Glycine (*r* = -0.56, p = 0.0001), Serine (*r* = -0.36, *p* = 0.02), Proline (*r* = -0.41, *p* = 0.007), Prolylproline (*r* = -0.44, *p* = 0.004) and hydroxyproline (*r* = -0.44, *p* = 0.003)

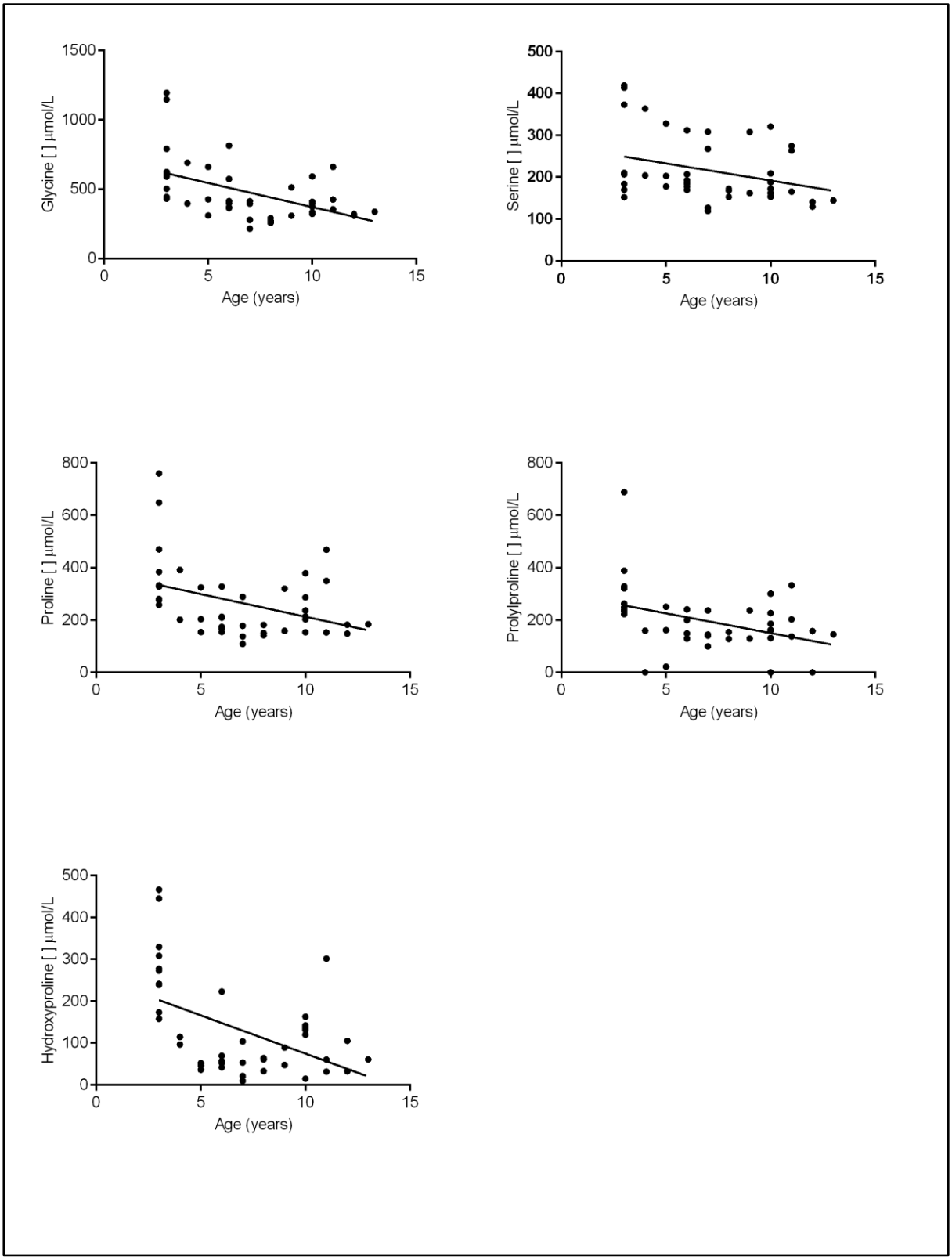
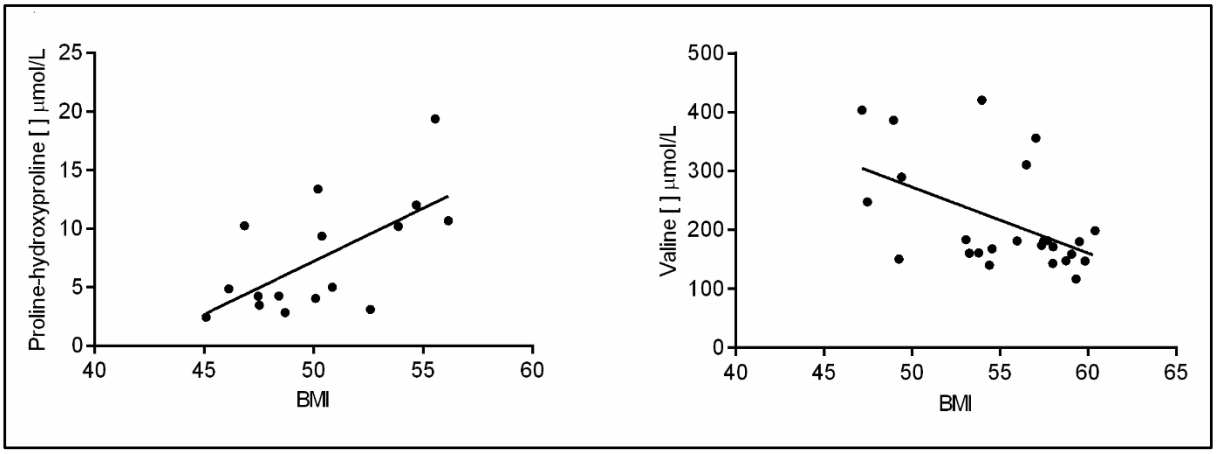


Figure 5-2. Scatterplots and linear regression showing the relationship between BMI and serum proline-hydroxyproline in female cheetahs (*r* = 0.41, *p* = 0.0073) and between BMI and serum valine in male cheetahs (*r* = -0.25, *p* = 0.01).



Urine samples were obtained from 26 (16 males and 10 females) of the 42 cheetahs. Data on the ages, BMIs, urine creatinine concentrations and urine specific gravities of these animals are shown in Table 5-4. In this subpopulation of animals there were no statistical differences between any of these variables.

Table 5-4. Summary of the age, BMI, urine creatinine concentrations and urine specific gravity data from 26 adult captive cheetahs in which urine amino acid concentrations were determined.

	Males (n=16)				Females (n=10)				
	Max	**Min**	**SD**	**Mean**	**Max**	**Min**	**SD**	**Mean**	**p-value**
Age (years)	11	3	2.56	6	10	3	3.03	6.4	0.72
BMI	60.4	47.2	4.49	54.94	56.16	46.1	3.6	52.1	0.099
Absolute creatinine (mmol/L)	83.44	18.91	21.34	50.18	99.69	13.88	26.36	45.42	0.617
Corrected creatinine (mmol/L)	75.12	16.36	19.3	50.80	68.01	16.36	19.0	44.27	0.404
Urine specific gravity	1.065	1.030	0.009	1.053	1.080	1.035	0.013	1.053	0.766

Thirty eight amino acids were identified and quantified in the cheetah urine samples, as shown in Table 5-5. Arginine was excreted at the highest mean concentration (740 µmol/L), followed by glutamine (437.1 µmol/L), alanine (397.1 µmol/L) and serine (331.1 µmol/L). As in the serum amino profiles, there were no significant differences in urine amino acid concentrations between male and female cheetahs. The fractional excretions (%FE) of the amino acids are shown in Table 5-6. The mean %FE of cystine was by far the highest at 21.92%, followed by hydroxylysine (6.34%), proline-hydroxyproline (5.49%), α-aminopimelic-acid (4.89%), β- alanine (4.05%), α-

aminoadipic-acid (3.98%), sarcosine (2.86%), cystathionine (2.41%) and glycine-proline (1.74%). The fractional excretion percentages of the remaining amino acids were all less than 1%.

Both urinary glycine and proline-hydroxyproline concentrations decreased significantly with age (r = -0.50, p = 0.009 and r = -0.41, p = 0.02 respectively) as shown in Figure 5-3. Several amino acid were also found to be excreted at significantly increased concentrations relative to BMI (Figure 5-4). These include alanine (r = 0.48, p = 0.01), total cysteine & cystine (r = 0.68, p = 0.0001), glycine (r = 0.62, p = 0.0008), aspartic acid (r = 0.64, p = 0.0004) and methionine (r = 0.61, p = 0.0009), while only 3-methylhistidine concentrations decreased significantly against increasing BMI values (r = -0.40, p = 0.04).

None of the serum and urine concentrations of individual amino acids correlated significantly with each other.

Figure 5-3. Scatterplots and linear regressions showing the relationship between age and urinary excretion of glycine (r = -0.50, p = 0.009) and proline-hydroxyproline (r = -0.46, p = 0.02).

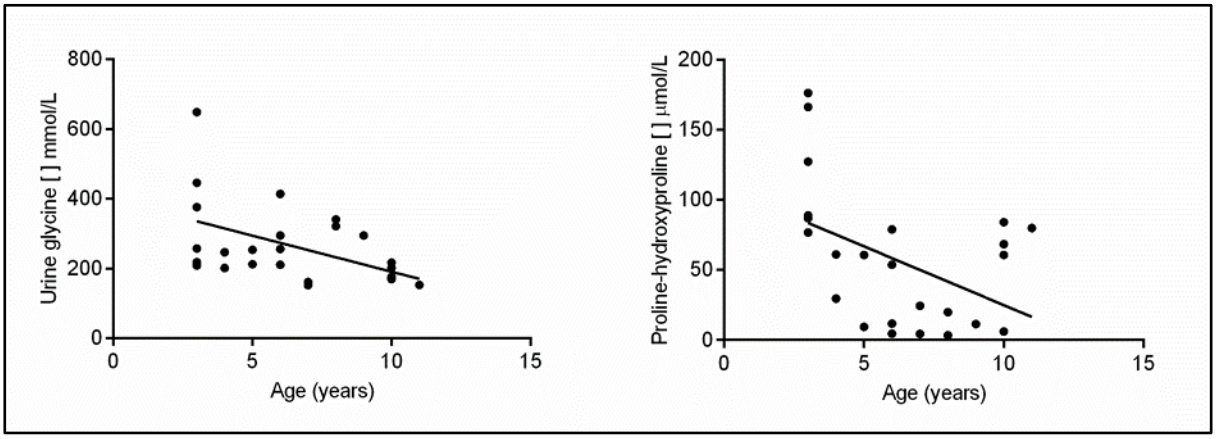


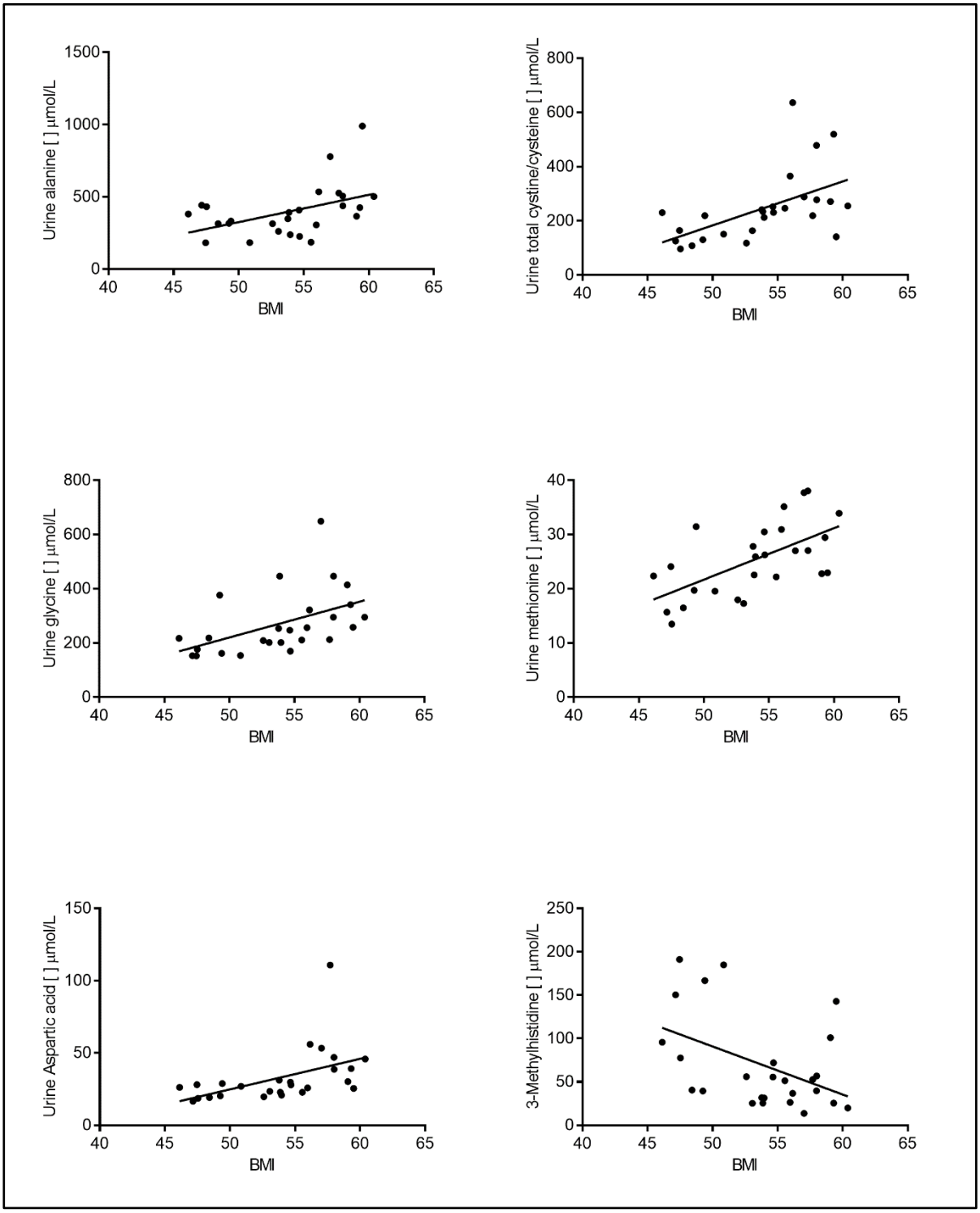
Table 5-5. Summary of the urine amino acid concentration data corrected to specific gravity in 26 adult cheetahs, listed in order from highest mean concentration to lowest (µmol/L).

Amino acid	Minimum	Median	Maximum	Mean	Std. Deviation
Arginine	295.3	640.8	2265.0	740.0	372.2
Glutamine	179.8	426.3	782.3	437.1	153.1
Alanine	181.7	373.1	989.5	397.1	179.6
Serine	191.7	331.1	605.2	331.3	81.9
Glycine	152.2	232.8	648.8	270.7	117.4
Cystine	96.62	230.7	636.1	245.1	129.8
Tryptophan	92.9	164.8	358.2	181.9	62.0
Lysine	85.1	145.4	298.7	156.2	51.9
Asparagine	43.1	114.5	353.9	124.0	61.9
Valine	26.1	62.5	493.0	95.0	106.1
Glutamic acid	57.3	91.4	180.9	91.4	26.7
Threonine	53.5	84.5	165.0	90.0	25.3
Histidine	46.7	81.2	197.7	84.2	29.1
Ornithine	22.1	52.2	282.9	70.3	60.9
3-Methylhistidine	13.7	52.0	191.1	69.6	53.8
α-Aminoadipic acid	2.9	41.2	176.0	64.1	55.0
Tyrosine	33.0	57.5	108.2	61.5	19.9
Proline-hydroxyproline	3.4	60.7	176.3	57.2	48.8
Cystathionine	12.5	24.3	376.4	48.3	76.7
Pyroglutamic acid	20.0	44.8	75.4	43.6	14.8
Proline	10.7	28.0	218.7	41.0	41.4
Citrulline	22.9	33.7	61.1	36.0	8.2
Aspartic acid	16.6	27.5	110.8	32.9	19.1
Leucine	15.5	25.1	70.2	28.8	12.1
β-Alanine	7.4	25.0	77.0	28.4	17.7
allo-Isoleucine	14.3	24.7	66.0	27.2	11.4
Phenylalanine	13.4	24.5	49.4	26.1	8.7
Methionine	13.5	25.0	38.1	25.3	6.8
Hydroxylysine	1.5	13.6	96.1	21.9	24.0
Glycine-Proline	5.4	15.7	31.3	15.6	6.2
Isoleucine	7.6	13.7	33.0	15.1	6.3
Prolylproline	0.0	1.4	203.0	10.9	39.9
Diaminopimelic acid	0.0	0.0	70.9	10.0	19.3
α-Aminobutyric acid	3.3	6.2	42.4	9.3	9.0
Cystine-Homocystine	0.0	4.2	79.4	9.2	17.4
Sarcosine	0.0	4.6	36.5	6.9	7.8
α-Aminopimelic acid	0.2	1.2	10.2	1.6	1.9

Table 5-6. Summary of the fractional excretion (FE%) of amino acids in 20 adult cheetahs, listed in order from highest to lowest.

Amino acid	Minimum	Median	Maximum	Mean	Std. Deviation
Cystine	4.55	20.24	54.81	21.92	13.99
Hydroxylysine	0.07	3.11	21.39	6.34	6.73
Proline-hydroxyproline	0.02	2.04	21.98	5.49	6.60
α-Aminopimelic-acid	0.02	3.36	22.37	4.89	5.86
β- Alanine	0.34	2.84	10.26	4.05	3.10
α-Aminoadipic-acid	0.25	2.83	10.37	3.98	3.47
Sarcosine	0.30	0.90	18.17	2.86	4.37
Cystathionine	0.11	0.80	14.02	2.41	4.34
Glycine-Proline	0.12	0.41	12.71	1.74	3.43
Asparagine	0.31	0.76	2.70	0.88	0.50
Tryptophane	0.14	0.58	2.12	0.76	0.56
Serine	0.26	0.63	1.47	0.72	0.34
Arginine	0.19	0.35	3.02	0.60	0.72
Aspartic-acid	0.14	0.40	1.74	0.49	0.36
Lysine	0.14	0.36	1.53	0.42	0.31
Ornithine	0.04	0.32	0.96	0.40	0.27
Citrulline	0.16	0.30	0.83	0.38	0.19
Tyrosine	0.09	0.33	0.85	0.37	0.21
α-Aminobutyric-acid	0.06	0.13	2.12	0.31	0.49
Alanine	0.06	0.25	1.32	0.29	0.27
Histidine	0.07	0.24	0.88	0.28	0.20
Threonine	0.09	0.28	0.55	0.28	0.12
Valine	0.04	0.12	1.89	0.25	0.41
Glutamic-acid	0.08	0.24	0.57	0.25	0.12
Glycine	0.10	0.26	0.40	0.25	0.08
Phenylalanine	0.06	0.17	0.45	0.21	0.11
Methionine	0.07	0.19	0.35	0.19	0.07
Pyroglutamic-acid	0.05	0.13	0.21	0.13	0.04
Glutamine	0.04	0.12	0.19	0.12	0.04
Isoleucine	0.03	0.07	0.16	0.08	0.04
Leucine	0.03	0.08	0.16	0.08	0.03
allo-Isoleucine	0.03	0.07	0.16	0.08	0.03
Proline	0.02	0.06	0.24	0.07	0.05
Prolylproline	0.00	0.01	0.14	0.02	0.04
Hydroxyproline	0.00	0.00	0.01	0.00	0.00

Figure 5-4. Scatterplots and linear regressions showing the significant correlations between BMI and corrected urine amino acids. Alanine (*r* = 0.48, p = 0.01), total cystine/cysteine (*r* = 0.68, p = 0.0001), glycine (r = 0.62, p = 0.0008), aspartic acid (*r* = 0.64, p = 0.0004), methionine (*r* = 0.61, p = 0.0009) and 3-methylhistidine (*r* = -0.40, p = 0.04).



5.4 Discussion

Serum or plasma amino acid profiles of large felids have, to our knowledge, not been reported in the literature. Serum alanine, lysine, ornithine and cystine values have been reported in a small number of caracals (*Caracal caracal*) and in all cases the concentrations were lower than those of the cheetahs in the present study (218). With the exception of asparagine and threonine, the cheetah serum amino acid concentrations were also generally higher than plasma values reported in domestic cats (216) (Table 5-7). Serum arginine and ornithine concentrations were substantially higher in cheetahs than in cats, with a 7.1 and 5.2-fold difference respectively. Alanine, glutamic acid, glutamine, hydroxyproline, lysine, tryptophan and citrulline were approximately twice as high in cheetah serum.

In most carnivorous species, blood amino acid concentrations appear to be carefully regulated, but in the postprandial period and during longer periods of fasting (> 2 days), larger variations have been noted (221). Arginine is considered an essential amino acid in carnivores as it is required in large quantities for conversion to ornithine, which in turn is required for ammonia detoxification in the urea cycle. Arginine is also required for creatine and nitric oxide synthesis (222). In domestic cats, a single arginine-free meal is known to result in severe, potentially fatal hyperammonaemia (223). It appears that cats have a limited ability to synthesize arginine *de novo* from glutamate and glutamine via citrulline and ornithine in the intestinal mucosa due to reduced activity of pyroline-5-carboxylate synthase and ornithine aminotransferase (73). Fortunately, arginine is replete in most animal tissues and under normal dietary conditions arginine deficiencies are unlikely to occur. Most large felids, like the cheetah, feed infrequently, consuming large quantities of food relative to their body size. It may therefore be necessary for large felids to maintain large serum arginine and or ornithine reserves to deal with the acute ammonia load resulting from such a large protein meal.

Five serum amino acid concentrations declined significantly relative to age in the cheetahs. These include serum glycine, serine, proline, prolylproline and hydroxyproline. Similar negative correlations for urine glycine and proline-hydroxyproline relative to age were demonstrated. The concentrations of these amino acids in some of the three-year-old cheetahs however appear to skew the data and if the data points of these three animals are removed, then the correlations are no longer significant. The higher concentrations of these related metabolites in urine and serum samples are, however, interesting. Hydroxyproline is present almost exclusively in collagen and is not re-utilised for collagen synthesis once it is broken down. Urine excretion of both free and peptide-bound hydroxyproline is therefore thought to reflect the breakdown of mature collagen (224). Both glycine and proline are also found at high concentrations in collagen. Urine hydroxyproline concentrations are often used to evaluate bone collagen metabolism in conditions like osteoporosis and osteomalacia (225). Since metabolic bone disease and nutritional

secondary hyperparathyroidism are commonly diagnosed in young growing large felids, due to either reduced calcium intake and/or hypovitaminosis D (124), it is possible that elevated hydroxyproline in these younger animals may result from subclinical nutritional deficiencies. At three years of age cheetahs are, however, sexually and skeletally mature and ideally serum and urine samples should be evaluated in younger animals that are still growing.

Comparison of the urinary amino acid excretion profiles cheetahs to those of other species is made difficult by the scarcity of studies in which quantitative urine amino acid data are reported, and by the fact that such studies generally report either 24-hr excretion values or creatinine corrected values. The proportional amino acid excretion patterns, however, provide some information for comparison. In domestic dogs alanine is the amino acid excreted at the highest concentration in urine, followed by aspartate, leucine, serine, lysine and glycine (226). In contrast, arginine was by far the most abundant amino acid in the cheetah urine at a mean concentration almost twice that of alanine. Other amino acids, ranking higher on the list compared to dogs included tryptophan and cystine. Felinine, the sulphur-containing amino acid frequently detected at high concentrations in domestic cats and other felid species (219) was not detected in any of the cheetah samples. 3-Methylhistidine, detected at high concentration in domestic cat urine (227), was only found at moderate concentrations relative to some of the other amino acids in the cheetahs.

Table 5-7. Comparative table of the mean serum amino acid concentrations (µmol/L) determined in this study and the plasma amino acids of domestic cats, reported in Pickett *et al* 1990 (228).

Amino acid	Mean serum AA [ ] in cheetahs	Mean plasma [ ] in domestic cats	Fold difference
Alanine	772	462	1.7
Asparagine	61	91	0.7
Aspartic-acid	35	28	1.2
Glutamic-acid	174	73	2.4
Glutamine	1624	648	2.5
Glycine	479	398	1.2
Histidine	157	116	1.4
Hydroxyproline	132	63	2.1
Isoleucine	86	63	1.4
Leucine	166	146	1.1
Lysine	187	108	1.7
Methionine	63	61	1.0
Ornithine	109	21	5.2
Phenylalanine	67	70	1.0
Proline	266	258	1.0
Serine	217	179	1.2
Threonine	154	173	0.9
Tryptophane	125	60	2.1
Tyrosine	84	57	1.5
Valine	220	164	1.3
Citrulline	41	18	2.3
Arginine	670	95	7.1

Serum concentrations of the dipeptide proline-hydroxyproline (also referred to as prolyl-hydroxyproline) increased relative to BMI values in female cheetahs, while serum concentrations of the branched-chain amino acid valine, decreased relative to BMI in males. The reason for these relatively weak associations is not yet clear.

The concentrations of several amino acids were positively correlated with BMI values in the cheetahs. This may simply reflect a more positive nitrogen balance in animals with higher BMIs. Non-essential amino acids such as alanine, aspartic acid and cystine may arguably be excreted more readily when protein-rich resources are abundant, but in the cheetahs, essential amino acids, such as methionine and conditionally essential glycine were also excreted at higher concentrations relative to BMI. Since elevated BMI values do not differentiate between increased body fat percentage and increased muscle mass, it is difficult to associate the changes in urine amino acids with a particular physiological process.

The only urinary amino acid metabolite that decreased relative to the cheetah BMI values, was 3-methylhistidine. The correlation coefficient (*r*) was, however, quite low at only -0.4, indicating a relatively weak relationship between these variables. The urinary excretion of this metabolite has been shown to be a useful marker of skeletal muscle protein catabolism in several species including humans, rats and the domestic cat (229). It is only produced by the donation of a methyl group to actin-bound histidine, and after protein degradation, it cannot be re-utilised and is excreted unchanged in the urine. Cheetahs with lower BMIs may be in a negative energy balance, releasing more 3-methylhistidine for urinary excretion. In some species, 3-methylhystidine is retained in the body in the form of β-alanyl-3-methylhistidine before it is excreted, making it less reliable as an indicator of skeletal muscle catabolism (230). Its use for this purpose in cheetahs will therefore have to be confirmed in future studies using radiolabelled 3-methylhistidine.

In healthy humans, more than 99% of the free amino acids are reabsorbed by the renal proximal tubules, resulting in fractional excretions of less than 1% of circulating amino acids (206,214). In the immediate postprandial period, amino acid excretion increases dramatically, but returns to baseline levels approximately 5 hours after a meal (231). The fractional excretion of dipeptides and modified amino acids is generally higher than that of standard free amino acids (232), possibly due to the lack of dedicated renal tubular transport mechanisms for these molecules. In the cheetahs, the dibasic amino acid cystine, had the highest fractional excretion of all the amino acids. The fractional excretion of cystine in cheetahs appears to be similar to those we previously recorded in healthy caracals (218). In wild felids, it is possible that only dedicated transmembrane transport mechanisms exist for cystine. It is likely that the high metabolic demands for cystine - for production of glutathione, taurine, co-enzyme A and general protein synthesis in felids - are easily met by the methionine and serine provided in animal tissues. The relatively high urinary excretion of cystine, together with increased activity of hepatic cysteine dioxygenase, may therefore limit the risk of cystine toxicity in these animals (233).

In this study we provide some foundational information on the serum and urine amino acid profiles of healthy captive cheetahs. Further research on the dynamic changes in serum amino acids after a typical meal should be evaluated and studies on the impact of dietary differences on the amino acid profiles in this species are warranted.

CHAPTER 6
SERUM FATTY ACIDS AND ACYLCARNITINES

6.1 Introduction

Fatty acids (FA) are a group of molecules within the lipid macronutrient class. They are carboxylic acids with a methyl end, a hydrocarbon chain and a carboxylic terminus. When compared to proteins and carbohydrates, FAs yield the most adenosine triphosphate (ATP) per gram, primarily through mitochondrial β-oxidation. They are hydrophobic and therefore transported in the circulating blood either as esters that are incorporated into triglycerides, or in a free non-esterified form attached to albumin. Besides providing a valuable source of energy, FAs also perform other vital functions in the body, including hormone production, cellular signalling as well providing structural components of biological membranes. Long-chain polyunsaturated fatty acids, with 20 carbon atoms, are the precursors of eicosanoids (prostaglandins, leukotrienes and thromboxanes), which have a wide range of regulatory, autocrine and paracrine effects. Polyunsaturated fatty acids (PUFAs) in the 20 to 22 carbon range are precursors of autacoids, including resolvins, lipoxins and neuroprotectins. Furthermore, fatty acids are known to play a role as modulators of gene transcription (234,235).

Most FAs have an even number of carbon atoms, as they are largely synthesised from two-carbon units. Fatty acids are classified according to the number and position of the double bonds in the carbon chain. These double bonds are absent in saturated fatty acids (SFAs), while mono-unsaturated fatty acids (MUFAs) have a single double bond and PUFAs have two or more double bonds in a pentadiene configuration. The melting point of FAs increases with the length of the hydrocarbon chain and decreases relative to the number of double bonds. Most of these double bonds have a *cis*-configuration under physiological conditions, resulting in a 30° bend in the carbon chain.

The most abundant FAs in both plants and animals have a chain length of between 16 and 18 carbon atoms and include palmitic, oleic, stearic and linoleic acids. Endogenous desaturation of FAs can only occur up to the $\Delta 9$ position (9^{th} carbon atom from the carboxyl end) and therefore certain PUFAs such as linoleic acid (LA) and α-linolenic acid (ALA) cannot be synthesised and must be included in the diet. Other longer chain PUFAs including eicosapentaenoic acid (EPA), arachidonic acid (AA) and docosahexaenoic acid (DHA) can be synthesized from LA and ALA to a limited extent, depending on the availability and activity of $\Delta 5$ and $\Delta 6$ desaturases.

Approximately 60 FAs have been identified in mammalian blood and tissue samples where the composition is largely influenced by genetic and dietary factors (236). The plasma or serum FA

profiles have been reported in several cat studies evaluating the effects of dietary PUFA supplementation (237), the impact of obesity and high fat diets (238), as well as profile changes in response to clinical conditions (239). Several human studies have shown that specific plasma FA concentrations reflect those consumed in the diet (240-242), correlating with dietary fat intake over a period of weeks to months (243). The FA composition of various tissue membrane phospholipids on the other hand, remain fairly constant, changing within a limited range in response to circulating FA concentrations (244). Plasma FA profiles therefore appear to provide a better indication of dietary fat composition than any of the tissue profiles.

Carnitine levels in the body are maintained by a combination of dietary intake, *de novo* synthesis and renal tubular reabsorption. Animal tissues contain relatively high concentrations of L-carnitine, while negligible quantities are available from plant sources. Carnitine is a small polar trimethylated amino acid that exists as two enantiomers (D-carnitine and L-carnitine), but only the L-isomer is physiologically important. It is able to form high-energy bonds with carboxylic acids, forming acylcarnitine derivatives. Carnitine is found throughout the body, but concentrated in muscle cells, and plasma or serum concentrations make up only around 0.1% of total body reserves (245). The endogenous carnitine pool is maintained within relatively narrow concentration limits and is primarily made up of L-carnitine as well as short, medium and long-chain acylcarnitines. Carnitine acts as a carrier of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation. This process results in the esterification of L-carnitine forming the acylcarnitine derivatives. The acylcarnitines include a group of approximately 40 esters of which acetylcarnitine is the most abundant. Under normal conditions the acylcarnitine to carnitine ratio is normally around 0.25, while values greater than 0.4 indicate abnormal mitochondrial function (245). L-carnitine also plays a vital role in regulating the availability of free coenzyme A (CoA). In the cytosol, fatty acids are activated via acyl-CoA synthase to form acyl-CoA. This thioester is then able to cross the outer mitochondrial membrane into the membrane space where it undergoes transesterification with L-carnitine to form free CoA and the corresponding acylcarnitine. This acylcarnitine is then transported across the inner mitochondrial membrane in exchange for free L-carnitine. Inside the mitochondrial matrix the acylcarnitine and available CoA undergo transesterification to form L-carnitine and the corresponding acyl-CoA, which is then available for β-oxidation. The intramitochondrial ratio of free CoA to acyl-CoA is reflected by the extramitochondrial ratio of acylcarnitine to L-carnitine (245).

Serum carnitine and acylcarnitine evaluations are typically undertaken in the screening of disorders of FA oxidation and some disorders of organic acid metabolism (246). Serum acylcarnitine profiles have been shown to be acutely responsive to variations dietary fatty acid intake (247). However, serum or plasma acylcarnitine profiles have not previously been reported

in any of the felid species and very little is known about the impact of dietary composition on the circulating concentrations.

The metabolism of FAs in felids appears to be quite unique in that they have been shown to have very low ∆6 desaturase activity (248). Numerous studies in domestic cats have therefore focused primarily on the impact of PUFA deficiencies (249-251), and more recently the relationship between obesity and fatty acid metabolism has been investigated (238). Clinical conditions attributed to altered fatty acid metabolism or deficiencies in cats include, hepatic lipidosis (252), pansteatitis (253), dystrophic mineralisation of the adrenal glands, degeneration of the testes, poor reproductive performance in females and hyperkeratosis of the skin (254). Other felids, such as the lion (*Panthera leo*) also seem to have low ∆-6 and ∆-8 desaturase activity, as they are unable to convert dietary sources of linoleic acid into dihomo-γ-linoleic acid and arachidonic acid (255).

The role of dietary fat in the incidence of various clinical abnormalities in cheetahs has been debated for some time, with some veterinarians advising that all visible fat be removed from meat fed to captive cheetahs. Other researchers have suggested that the higher incidence of pancreatitis in the North American cheetahs and the unusual splenic myelolipomas and pulmonary lipid accumulations, seen in captive cheetahs, may be the result of excessive dietary fat (10). Not all fats are, however, equal and the actual FA composition of the diets fed to captive cheetahs may be important for optimal health. Not only does the whole-body FA composition of domestic prey species vary tremendously, but the fatty acid composition in different organs meats is variable within a species (256). Given the range of diets fed to captive cheetahs, the fatty acid composition of diets are likely to differ substantially and may be vastly dissimilar from the FA intake they have adapted to in the wild. In one study, lower proportions of oleic acid, α-linolenic acid and all the C22 PUFAs were demonstrated in the liver fatty acid fractions of a single captive cheetah and compared to those of two free-ranging cheetahs (257). The authors of this study admitted that these observed differences could be due to the advancing age, the alternative diet or reduced freshness of the food fed to the captive animal. They however suggested that their results provided circumstantial evidence of reduced ∆-6 desaturase activity in the cheetah. The same group of researchers also described a return to oestrus and improved coat and skin condition in two female cheetahs supplemented with dihomo-γ-linoleic acid and eicosapentaenoic acid (258). Serum fatty acid fractions were reported in four cheetahs suffering from hyperlipidaemia as well as 28 captive cheetahs housed at two zoological facilities in North America. The authors concluded that similar to domestic cats and lions, the cheetah fatty acid profiles showed evidence of reduced ∆-6 and ∆-8 desaturase activity (76). The serum or plasma fatty acid profiles of free-ranging cheetahs on a natural diet have however not been evaluated.

In this study we evaluated the serum FA, carnitine and acylcarnitine profiles of both captive and free-ranging cheetahs. Given that the free-ranging cheetahs largely consume naturally occurring non-domestic prey species and rarely suffer from any of the clinical abnormalities that are so common in captive cheetahs, we would expect their serum FA and carnitine profiles to serve as reliable, healthy controls against which the serum levels of captive cheetahs can be evaluated. Furthermore the number of samples obtained would also allow us to assess the impact of ageing on the serum FA and carnitine profiles, as well as evaluate the profile differences between males and females.

6.2 Materials and methods

6.2.1 Samples

Blood samples were collected from 35 adult captive cheetahs (14 females and 21 males) at the AfriCat Foundation near Otjiwarongo in Namibia as described in Chapter 5 during their annual health assessments in July 2013. These animals were fed a diet consisting mostly of muscle meat from eviscerated and exsanguinated donkeys, supplemented with a multivitamin and mineral powder (Predator Powder ®, Healthtech, South Africa). Additional samples were collected by researchers from the Liebniz Institute of Zoo and Wildlife Research (IZW) from 44 free-ranging sub-adult and adult cheetahs (36 males and 8 females) on communal and commercial farmland in the Khomas and Omaheke districts in central Namibia.

The free-ranging cheetahs were captured in scent-bated box traps placed at known cheetah marking trees between July 2012 and May in 2013. Once secured in the box traps, they were immobilised by remote intramuscular injection using a combination of 0.06 mg/kg medetomidine hydrochloride (Medetomidine 10 mg/ml, Kyron Laboratories, South Africa) and 3.2 mg/kg ketamine (Ketamine 1G, Kyron Laboratories, South Africa). The ages of the free-ranging cheetahs were estimated by using the key for body size established by Caro (1994) (5) by evaluating shoulder height, appearance of the mane and physical lesions such as elbow calluses, scars as well as dental wear. Body mass indices for the captive cheetahs were determined as described in Chapter 2. Unfortunately BMI data were not available for the free-ranging animals.

Within 15 to 35 minutes of immobilisation, blood was collected aseptically by different operators, using slightly different methods. In the captive animals, blood was collected from a jugular vein with a 20ml syringe and 18 gauge needle and then transferred into 6ml BD Vacutainer® tubes (Becton, Dickinson and Company, South Africa), while in the free-ranging cheetahs the blood was collected from the cephalic vein directly into the 6ml BD Vacutainer® tubes through a 21 gauge Vacutainer® needle. All the blood samples were allowed to clot on ice in a cooler box. Samples

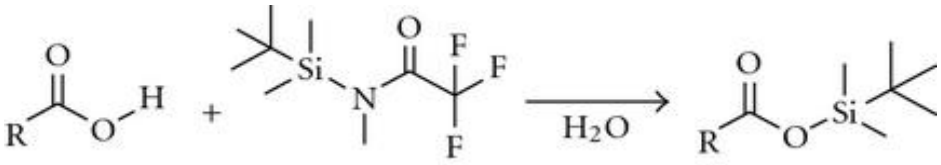
were centrifuged within 4 hours at either 1500g for 10 minutes (captive cheetahs) or within 4 to 12 hours at 400g for 15 minutes (free-ranging cheetahs), after which the serum was pipetted off and immediately frozen at -20°C. The samples were then transported on dry ice to the laboratory in November 2013 and kept at -80°C until analysis in January 2014.

6.2.2 Fatty acid analyses

6.2.2.1 Sample preparation and reagents

After thawing the samples on ice, they were subject to both acidic and alkaline hydrolysis and then extracted into hexane as described by (259) for the analysis of very long-chain fatty acids (VLCFA). The protocol was, however, modified for the additional quantification of long-chain fatty acids (LCFA) by adding an additional stable isotope standard (eicosanoic acid-d_{39}) to the internal standard solution at a concentration of 50 µmol/l. Derivatisation was achieved with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) as described by the same authors.

Figure 6-1 MTBSTFA derivatization reaction



Eicosanoic-d_{39} acid was obtained from Sigma-Aldrich Pty LTD (Kempton Park, South Africa). The very long chain fatty acid (VLCFA) stable isotope standards: C26:0-d_4, C24:0-d_4, C22:0-d_4, pristanic acid-d_3 and phytanic acid-d_3 were obtained from Dr. Herman ten Brink (VU Medical Centre, Amsterdam, The Netherlands) (http://www.vumc.nl/metabool/index.html). Other reagents were obtained from Larodan (Karolinska Institutet Science Park, Retzius väg 8, SE-171 65 Solna, Sweden), Merck Pty LTD (Modderfontein, South Africa) and Regis Technologies, Inc (Morton Grove, IL, USA).

6.2.2.2 Gas chromatography-mass spectrometry

The GC-MS analysis of the serum LCFAs and VLCFAs, was conducted on a Hewlett Packard HP6890 series gas chromatography (GC) system with an Agilent 5973N Mass selective detector fitted with an electron ionization (EI) ion source, an Agilent Technologies 7683 autosampler and a 7683B injector. The GC system was fitted with a Phenomenex GC FocusLiner liner for HP,

split/splitless, w/wool, single taper, 4 mm ID x 78.5 mm L x 6.3 mm OD (part number AG0 4680) and a CPSil19 capillary column (25 m×0.25 mm×0.20 µm; Varian). The injection system was used in the splitless mode and kept at 300°C. The interface to the mass selective detector was set at 290°C.

Each sample was injected initially for the detection of VLCFAs in the selected-ion monitoring (SIM) mode and a second time for the detection of the LCFAs in scan mode. For the detection of VLCFAs, the GC separation of the analytes was achieved using the following column temperature program: initial temperature 60°C for 1 min, increase to 240°C at a rate of 30°C/min, further increase to 270°C at a rate of 10°C/min, final increase to 300°C at 4°C/min and 5 min isothermal at the latter level. The mass spectrometer was operated at 70 eV in the SIM mode. A dwell time of 100 ms and a relative EM voltage of 400 V higher than that in the scanning mode were chosen for each ion monitored.

For the detection of LCFAs, the GC separation of the analytes was achieved using the following column temperature program: initial temperature 50°C for 1 min, increase to 270°C at a rate of 30°C/min, further increase to 320°C at a rate of 4°C/min and 5 min isothermal at the latter level. The mass spectrometer was operated at 70 eV in the SCAN mode (scan range of 50–650 amu). Helium was used as the carrier gas at a constant flow rate of 1.0 ml/min for both VLCFA analysis and total FA analysis. MS conditions were as follows: EI mode, ion source temperature, 200°C, multiplier voltage, 1.182 V, solvent delay 5 min.

The FAs, were quantified with Agilent MSD ChemStation E02.00 software. A linear regression was used to calibrate the identification and quantification of the FAs. The linearity, lower detection limit and maximum detection limit were determined for all the FAs. A quality control (QC) standard mixture of human control serum was run with each batch. The human control was prepared by aliquoting 100 µl portions of a normal range pooled serum into screw-cap vials. The mean and standard deviation for each QC sample was calculated with a minimum of 20 between-run values. Control values that fell within standard deviations of the mean were considered acceptable.

6.2.3 Acylcarnitine analyses

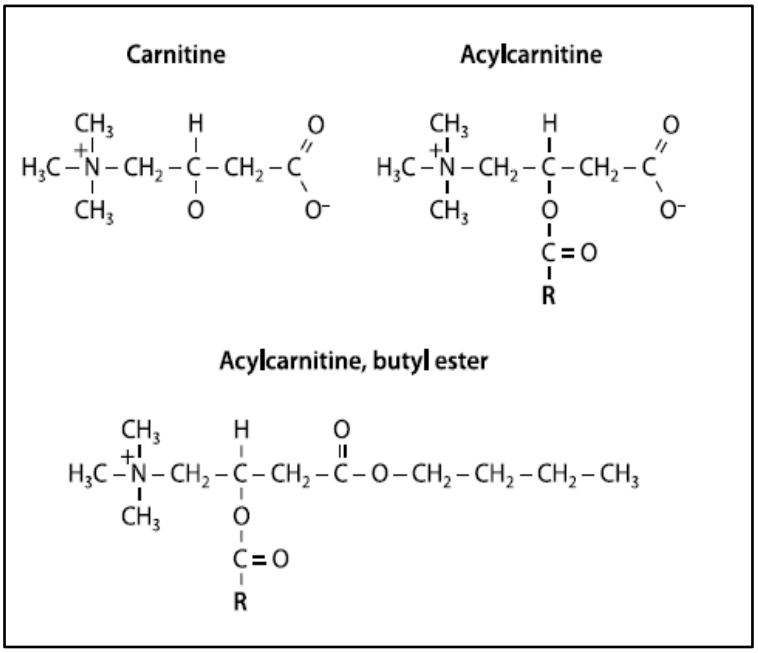
6.2.3.1 Sample preparation and reagents

Acylcarnitines were extracted and derivatized as described by Matern (260). Extraction was achieved with a methanol solution containing labelled acylcarnitine isotopes of various chain lengths at defined concentrations. Samples were then derivitized with *n*-butanol HCl.

The acylcarnitine standards were obtained from Sigma-Aldrich Pty LTD (Kempton Park, South Africa). Stable isotope standards: were purchased from Dr. Herman ten Brink (VU Medical Centre, Amsterdam, The Netherlands) (http://www.vumc.nl/metabool/index.html). Other reagents were supplied by Merck Pty LTD (Modderfontein, South Africa) and Regis Technologies, Inc (Morton Grove, IL, USA).

The stable isotope carnitine mixture (410 µl) was pipetted into micro-centrifuge tubes to which 10 µl of serum was added. The mixture was centrifuged at 13 000g for 2 minutes after which the supernatant was pipetted into a clean micro-centrifuge tube. The tubes were then placed in a nitrogen dryer at 65°C for approximately 45 minutes. Once dry, 200 µl 3N buthanolic HCl was added and the tubes were then capped and placed in an incubator, set at 65°C for 15 minutes. The tubes were then placed in a nitrogen dryer for approximately 20 minutes and once dry, 100 µl of the mobile phase (acetonitrile:water [50:50] with 1% acetic acid) was added. The samples were then transferred to LC vials for analysis.

Figure 6-2. Structures of carnitine, acylcarnitine and butylated acylcarnitine. The R represents the acylcarnitine species with up to 18 carbons.



6.2.3.2 Liquid chromatography-tandem mass spectrometry

Controlled by the MS-MS software, the Agilent 1200 Series LC system pump operates isocratically using 80% acetonitrile:water at 40 µl/min. A 50 × 1 mm C18 column (Keystone) is used between the pump and autosampler to provide back-pressure. The syringe/system flush solution is the 80% acetonitrile:water used as the mobile phase. The autosampler (Agilent 1200 Series G1367A Well-Plate Autosampler) is connected directly to the MS-MS (Agilent 6460 Triple Quadrupole LC/MS) electrospray source. The injected sample volume is 5 µl. A 0.5-U Peek filter is installed between the autosampler and electrospray interface source. The filter end fitting is discarded and replaced after approximately 200 injections.

Acylcarnitine analysis was done on the Agilent 6420 Triple Quad MS in the ESI mode (Delta EMV=400 V) connected to the Agilent 1260 HPLC. Acylcarnitine analysis is performed as a precursor scan in positive ion mode. Q1 is set to scan a mass range from m/z 200 to 500, while Q3 is set to determine a precursor ion of m/z 85. The MS-QQQ settings were as indicated in Table 6-1.

Table 6-1. MS-QQQ settings for the acylcarnitine analysis

Prod Ion	Scan from to		Scan Time	Frag made	Frag (V)	CE (V)	Cell Acc (V)	Polarity
85	200	500	800 S	Fixed	135	20	7	+

Gas temp-280 C, Gas flow-7.5 l/min, Nebulizer-28 psi, capillary-3500 V, injection volume-µl. The method is optimized for a mixture of butylesters containing d_3-C2, d_3-C3, d_3-C4, d_3-C8, d_3-C12, and d_3-C16 acylcarnitine. The sample queue is entered into the MS-MS instrument's software according to the manufacturer's instructions.

Table 6-2. Relevant acylcarnitine species included in a typical acylcarnitine analysis

Mass[a]	Acylcarnitine species	
218	C0	Free carnitine
260	C2	Acetylcarnitine
274	C3	Propionylcarnitine
287	FIGLU	Formiminoglutamate
288	C4	Butyryl-/Isobutyrylcarnitine
302	C5	Isovaleryl-/2-Methylbutyrylcarnitine
304	C4-OH	3-Hydroxybutyrylcarnitine
314	C6:1	3-Methylglutaconylcarnitine
316	C6	Hexanoylcarnitine
318	C5-OH	3-Hydroxyisovaleryl-/2-Methyl-3-hydroxybutyrylcarnitine
330	C7	Heptanoylcarnitine
342	C8	Octanoylcarnitine
360	C3-DC	Malonylcarnitine
360	C8-OH	3-Hydroxyoctanoylcarnitine
368	C10:2	Decadienoylcarnitine
370	C10:1	Decenoylcarnitine
372	C10	Decanoylcarnitine
374	C4-DC	Methylmalonyl-/succinylcarnitine
388	C5-DC	Glutarylcarnitine
388	C10-OH	3-Hydroxydecanoylcarnitine
400	C12	Dodecanoylcarnitine
402	C6-DC	3-Methylglutarylcarnitine
416	C12-OH	3-Hydroxydodecanoycarnitine
424	C14:2	Tetradecadienoylcarnitine
426	C14:1	Tetradecenoylcarnitine
428	C14	Myristoylcarnitine
444	C14-OH	3-Hydroxytetradecanoylcarnitine
456	C16	Palmitoylcarnitine
470	C16:1-OH	3-Hydroxyhexadecenoylcarnitine
472	C16-OH	3-Hydroxyhexadecanoylcarnitine
480	C18:2	Linoleylcarnitine
482	C18:1	Oleylcarnitine
484	C18	Stearoycarnitine
498	C18:1-OH	3-Hydroxyoleoylcarnitine
500	C18-OH	3-Hydroxystearoylcarnitine

[a] *Mass of the molecular ion of the butylated acylcarnitine esters*

6.2.4 Statistical analyses

Standard range analysis, using the MSD ChemStation E02.00 software with a linear regression curve fit was used to calibrate the identification and quantification of the fatty acids. The results were exported to Microsoft Excel® for further analysis.

Data was tested for normality using the D'Agostino and Pearson omnibus (K2) normality test. The concentrations of palmitic acid, stearic acid, nonadecanoic acic, arachidic acid, behenic acid, lignoceric acid, pristanic acid, phytanic acid, total SFA, LA, γ-linonenic acid, arachidonic acid (AA),

eicosadienoic acid, eicosapentanoic acid, total ω-6, total PUFA and total FA concentrations were normally or near-normally distributed. The concentrations of myristic acid, heptadecanoic acid cerotic acid, hypogeic acid, palmitoleic acid, oleic acid, total MUFA, SFA:PUFA, ω-6:ω-3 and the desaturase index had to be logarithmically transformed to meet normality requirements. None of the acylcarnitine concentrations were normally distributed and the data was left untransformed for non-parametric analyses.

Spearman's rho correlation coefficients were used to evaluate the relationship between age, body mass index (BMI) and the various fatty acid. Unpaired t-tests were used for comparisons between FA concentrations in males versus females and between the captive and free-ranging individuals. Mann-Whitney U tests were used to compare the acylcarnitine concentrations in captive versus free-ranging cheetahs. All analyses were performed with GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, California, USA). All statistical tests were 2-tailed and significance was defined as $P<0.05$ in all cases, however, multiple comparisons were run with a Bonferroni adjustment.

6.3 Results

The captive cheetahs in this study, were significantly older (mean age = 7.14 years) than the free-ranging cheetahs (mean age = 3.72 years) ($p < 0.0001$). The free-ranging cheetahs also had a significant male sex bias (34 males versus only 9 females). The influence of sex and age on the various parameters were therefore evaluated separately in the two populations. In the captive cheetahs weak negative correlations were found only for eicosadienoic acid (C20:2ω6) ($r = -0.40$; $p = 0.016$) and arachidic acid (C20:0) ($r = -0.40$; $p = 0.02$) as shown in Figure 6-3. In the free-ranging cheetahs, weak positive correlations were noted for arachidonic acid (C20:4ω6) ($r = 0.43$; $p = 0.004$), stearic acid (C18:0) ($r = 0.34$; $p = 0.03$) and heptadecanoic acid (C17:0) ($r = 0.34$; $p = 0.02$), while hypogeic acid (C16:1ω9) decreased significantly relative to age ($r = -0.32$; $p = 0.03$), as shown in Figure 6-4. Body mass index scores did not significantly correlate with any of the serum fatty acid concentrations in the captive animals.

Figure 6-3. Scatterplots and linear regressions showing the relationship between serum fatty acids that correlated significantly with the age of captive cheetahs. Eicosadienoic acid (*r* = -0.40; p = 0.016) and arachidic acid (*r* = -0.40; p = 0.02)

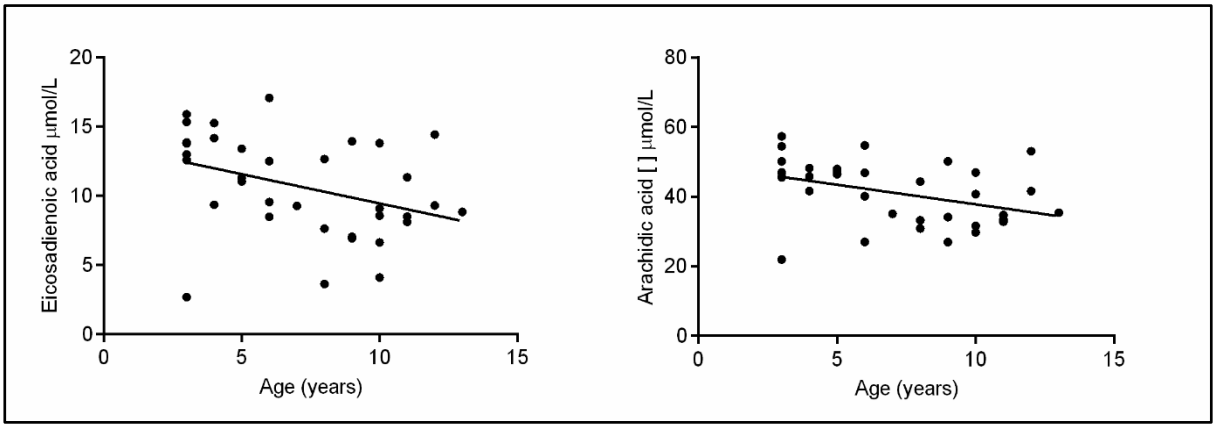
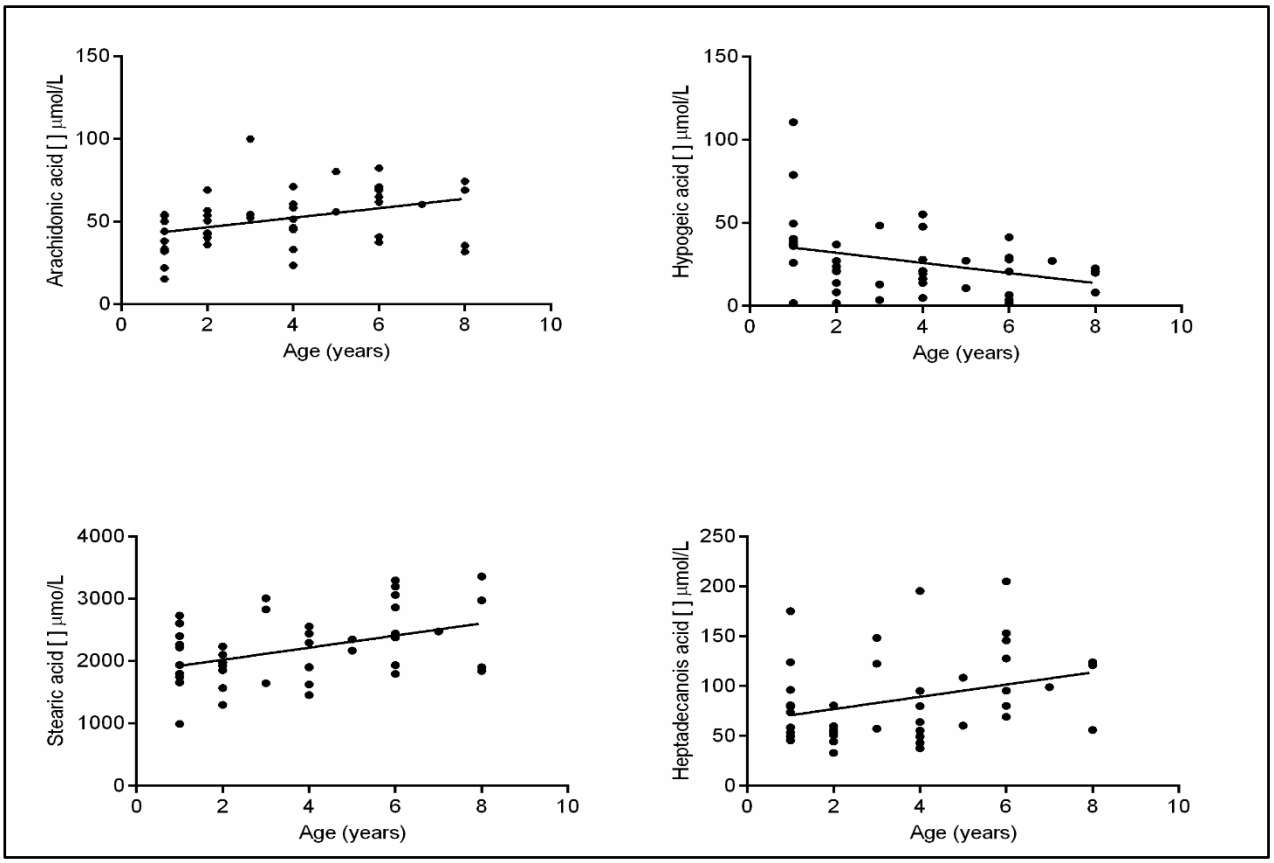


Figure 6-4. Scatterplots showing the relationship between serum fatty acids that correlated significantly with the age of free-ranging cheetahs. Arachidonic acid (*r* = 0.43; p = 0.004), stearic acid (*r* = 0.34; p = 0.03) and heptadecanoic acid (*r* = 0.34; p = 0.02), while hypogeic acid decreased significantly relative to age (*r* = -0.32; p = 0.03).



Comparative mean FA concentrations differed dramatically between the captive and free-ranging cheetahs, as shown in Table 6-3. The captive cheetahs had significantly higher total long-chain fatty acid (C12 to C20) concentrations than the free-ranging cheetahs ($p < 0.0001$). The total SFA concentrations were slightly higher in the free-ranging cheetahs, but this difference was not statistically significant ($p = 0.07$). In contrast both the total MUFA and PUFA serum concentrations were significantly higher in the captive animals ($p < 0.0001$ in both cases). Total ω-3 and ω-6 concentrations were significantly higher in the captive cheetahs ($p < 0.0001$), but the ω-6:ω-3 ratio was similar in both groups ($p = 0.2$).

Stearic acid (C18:0) was the most abundant fatty acid in the serum of both the captive and free-ranging cheetahs, with similar mean concentrations of 2028.9 µmol/L and 2127.4 µmol/L respectively ($p = 0.37$). This was followed by palmitic acid (C16:0) which was significantly higher in the free-ranging cheetahs ($p = 0.003$). The mean polyunsaturated omega-6 fatty acid, linoleic acid (C18:2ω6) was the third most abundant FA and more than three-fold higher in the captive cheetahs than in their free-ranging counterparts ($p < 0,0001$). Similarly, the mean concentration of the mono-unsaturated oleic acid (C18:1ω9), was more than four-fold higher in the captive cheetahs ($p < 0.0001$). Of the remaining MUFAs and PUFAs, only hypogeic acid (C16:1ω9) and arachidonic acid (C20:4ω6) were detected at significantly higher concentrations in the free-ranging cheetahs. All the other unsaturated fatty acids were significantly more abundant in captive cheetah serum ($p < 0.0001$).

Odd-chain fatty acids were also present in the serum of most of the cheetahs. Heptadecanoic acid (C17:0) was, however, far more abundant than nonadecanoic acid (C19:0) and detected at significantly higher concentrations in the free-ranging cheetahs than in those in captivity ($p < 0.0001$). The concentrations of nonadecanoic acid were moderately higher in the captive animals ($p = 0.01$).

The only ω-3 PUFA detected in any of the cheetah serum was eicosapentaenoic acid (EPA - C20:5ω3), while its presumed precursors, namely α-linolenic acid (C18:3ω3) and the end product of ω-3 desaturation/elongation, docosahexaenoic acid (DHA – C22:6ω3), were not detected in any of the samples. The free-ranging cheetahs had significantly lower EPA concentrations than those in captivity ($p < 0.0001$).

The branched-chain fatty acids, pristanic acid and phytanic acid, were both detected at higher concentrations in the free-ranging cheetahs ($p < 0.0001$) as shown in Table 6-5. This was also true for the very long-chain fatty acids, behenic (C22:0) and lignoceric acid (C24:0) ($p < 0.0001$). The serum fatty acid with the longest chain length, namely cerotic acid (C26:0), was detected at similar concentrations in both the captive and free-ranging cheetahs ($p = 0.511$). Out of the branched and very long-chained fatty acids, only pristanic acid concentrations declined

significantly relative to age in the captive cheetahs (r = -0,40; p = 0.009). The pristanic acid to phytanic acid ratio also declined significantly relative to age in the captive animals (r = -0.54; p = 0.0008) (Figure 6-5), while no such decline was found in the free-ranging cheetahs. As with the LCFAs, BMI values did not correlate significantly with any of the VLCFAs or BCFAs.

Figure 6-5. Scatterplot and linear regressions showing: On the left - the relationship between serum pristanic acid concentrations and the age of captive cheetahs (r = -0,40; p = 0.009) and on the right - the relationship between the pristanic acid:phytanic acid ratio and the age of captive cheetahs (r = -0.54; p = 0.0008).

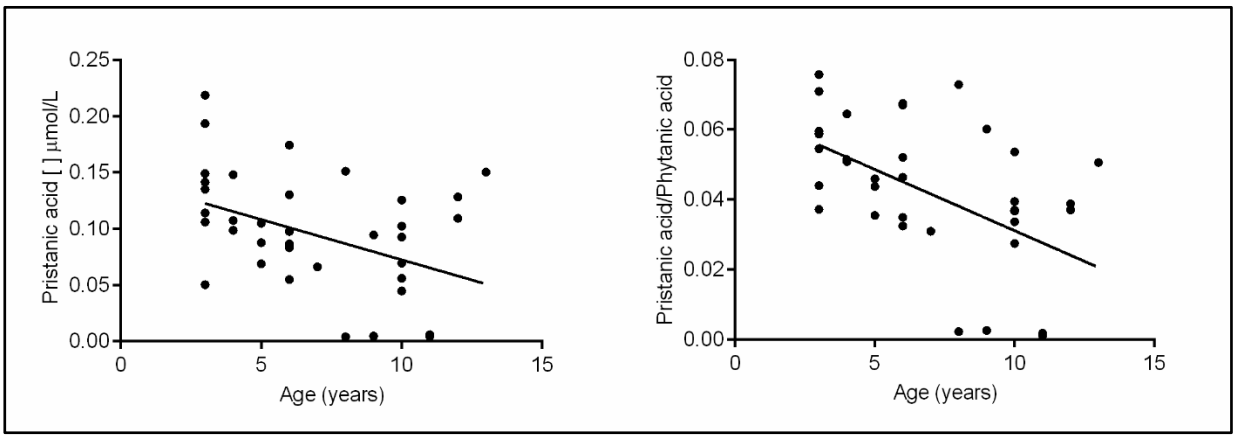


Table 6-3. Serum long-chain fatty acid concentrations in µmol/L (means and standard deviations) of both captive (n = 35) and free-ranging (n = 43) cheetahs

		Captive		Free-ranging		
		Mean	**SD**	**Mean**	**SD**	***p*-value**
Age (years)		7.14	3.2	3.72	2.2	<0.0001*
Myristic acid	C14:0	31.16	16.2	34.38	19.7	0.22
Palmitic acid	C16:0	1182.3	242.7	1385.2	366.0	0.003
Heptadecanoic acid	C17:0	45.10	9.9	84.67	42.8	<0.0001*
Stearic acid	C18:0	2028.9	451.4	2127.4	520.3	0.37
Nonadecanoic acid	C19:0	0.05	0.03	0.03	0.02	0.01
Arachidic acid	C20:0	41.05	9.1	30.93	7.5	<0.0001*
Total SFA		**3344**	**685.8**	**3675**	**881.4**	**0.07**
Hypogeic acid	C16:1ω9	5.87	6.7	26.10	21.1	<0.0001*
Palmitoleic acid	C16:1ω7	46.97	19.3	18.35	17.4	<0.0001*
Oleic acid	C18:1ω9	821.44	362.4	178.68	210.8	<0.0001*
Total MUFA		**874.30**	**374.7**	**223.10**	**212.9**	**<0.0001***
Linoleic acid	C18:2ω6	1049.79	329.3	294.2	125.9	<0.0001*
γ-Linolenic acid	C18:3ω6	86.58	58.1	8.83	6.6	<0.0001*
Eicosadienoic acid	C20:2ω6	10.90	3.4	0.83	0.9	<0.0001*
Dihomo-γ-linolenic acid	C20:3ω6	ND	-	ND	-	-
Arachidonic acid	C20:4ω6	35.91	8.7	52.19	17.8	<0.0001*
Total ω6		**1183**	**355**	**357**	**137**	**<0.0001***
α-Linolenic acid	C18:3ω3	ND	-	ND	-	-
Eicosatetraenoic acid	C20:4ω3	ND	-	ND	-	-
Eicosapentaenoic acid	C20:5ω3	14.69	4.6	5.54	4.7	<0.0001*
Docosahexaenoic acid	C22:6ω3	ND	-	ND	-	-
Total ω3		**14.69**	**4.6**	**5.54**	**4.7**	**<0.0001***
Total PUFA		**1198**	**358.5**	**369.6**	**144.8**	**<0.0001***
ω6:ω3		84.0	24.2	281	462	0.2
SFA:PUFA		3.01	0.8	11.14	2.9	<0.0001*
Desaturase index		0.41	0.17	0.09	0.10	<0.0001*
Total FA		**5470**	**1253**	**4312**	**993**	**<0.001***

** p < 0.0019, Bonferroni adjustment 0.05/27 tests. **ND = not detected*

Table 6-4. Proportions of individual and total SFAs, MUFAs and PUFAs in captive and free-ranging cheetahs as reported in the present study are compared to fatty acids in the plasma phospholipid fraction of captive cheetahs as reported by Bauer *et al* (76).

		Present study captive cheetah mean %	Present study free-ranging cheetah mean %	Bauer *et al* captive cheetah (n = 28) mean %
Myristic acid	C14:0	0.6	0.8	NR
Palmitic acid	C16:0	21.8	32.5	NR
Heptadecanoic acid	C17:0	0.8	2.0	NR
Stearic acid	C18:0	37.5	49.8	NR
Nonadecanoic acid	C19:0	0.001	0.001	NR
Arachidic acid	C20:0	0.8	0.7	NR
Total SFA		**61.7**	**86.1**	**48.4**
Hypogeic acid	C16:1ω9	0.1	0.6	NR
Palmitoleic acid	C16:1ω7	0.9	0.4	NR
Oleic acid	C18:1ω9	15.2	4.2	NR
Total MUFA		**16.1**	**5.2**	**13.0**
Linoleic acid	C18:2ω6	19.4	7.0	12.5
γ-Linolenic acid	C18:3ω6	1.6	0.2	NR
Arachidonic acid	C20:4ω6	0.7	1.2	15.0
Eicosapentaenoic acid	C20:5ω3	0.3	0.1	1.4
Eicosadienoic acid	C20:2ω6	0.2	0.0	0.4
Total PUFA		**22.1**	**8.7**	**34.9**
Total ω3		0.3	0.1	4.9
Total ω6		21.8	8.5	30.0
PUFA:SFA		0.36	0.10	0.72

NR = not reported

Table 6-5. Serum branched-chain and very long-chained fatty acids concentrations in µmol/L (means and standard deviations) of captive (n = 35) and free-ranging (n = 43) cheetahs.

		Captive		Free-ranging		
		Mean	**SD**	**Mean**	**SD**	**p-value**
Pristanic-acid		0.09	0.05	0.73	0.81	<0.0001*
Phytanic-acid		2.34	0.74	13.45	8.70	<0.0001*
Behenic acid	C22:0	29.91	4.59	41.39	13.30	<0.0001*
Lignoceric acid	C24:0	36.30	6.11	57.53	17.44	<0.0001*
Cerotic acid	C26:0	0.89	0.42	0.83	0.43	0.511

p < 0.01, Bonferroni adjustment 0.05/5 tests.

The serum carnitine and acylcarnitine concentrations for the 35 captive and 43 free-ranging cheetahs are shown in Table 6-6. The concentrations of isovaleryl-carnitine, 2-methylbutyryl-carnitine and pyvaroyl-carnitine cannot be quantified separately using GC-MS and their collective concentration are therefore reported as C5-carnitine. Similarly, butyrylcarnitine and isobutyrylcarnitine (C4), have identical molecular masses and cannot be distinguished on GC-MS.

Only carnitine and the C5-carnitines were detected in all the samples. Most of the other acylcarnitines were either not detected at all (C12 and C18), or only detected in one or two serum samples from the captive cheetahs. In contrast the acylcarnitine with the lowest detection frequency in the free-ranging cheetahs was hexanoylcarnitine (C6) (detected in 9 of 44 individuals). Carnitine concentrations were significantly higher in the captive cheetahs compared to the free-ranging cheetahs ($p = 0.0001$). In contrast, all the acylcarnitines, except for C5, were detected at significantly higher concentrations in the free-ranging cheetahs. Consequently, both the acetylcarnitine to carnitine ratio and the acylcarnitine to carnitine ratios were significantly higher (7.8 fold and 5.3 fold respectively) in the free-ranging cheetahs ($p < 0.0001$).

Table 6-6. Mean serum carnitine and acylcarnitine concentrations with standard deviations in captive and free-ranging cheetahs.

	Captive (n = 35)			Free-ranging (n = 44)			
	No. detected in	**Mean (µmol/L)**	**Std. Dev.**	**No. detected** in	**Mean (µmol/L)**	**Std. Dev.**	**p-value**
Carnitine (C0)	35	15.44	2.98	44	12.55	2.61	<0.0001*
C2	2	0.32	1.42	22	1.74	1.98	<0.0001*
C4	1	0.03	0.20	10	0.14	0.30	0.01
C5	35	0.22	0.12	44	0.27	0.13	0.07
C6	1	0.001	0.008	9	0.01	0.023	0.012
C12	0	ND		11	0.007	0.013	0.0006*
C12:1-OH	1	0.001	0.003	26	0.045	0.063	<0.0001*
C16	2	0.001	0.004	17	0.007	0.011	0.0003*
C18	0	ND		18	0.009	0.013	<0.0001*
Acetylcarnitine:Carnitine ratio	2	0.018	0.079	22	0.142	0.161	<0.0001*
Acylcarnitine:Carnitine ratio	35	0.034	0.085	44	0.179	0.172	<0.0001*

*ND – not detected, *p < 0.0045, Bonferroni adjustment 0.05/11*

6.4 Discussion

The serum fatty acid, carnitine and acylcarnitine profiles of captive cheetahs contrasted dramatically with those in the free-ranging cheetahs, reflecting significant differences in dietary FA composition and/or FA metabolism. These findings are not entirely unsurprising given the differences in the diets consumed by these two groups of animals. The donkeys fed to the captive cheetahs in this study were eviscerated after slaughter and the internal organs and intra-abdominal fat discarded as they easily spoil, even when refrigerated. Their diet therefore consisted primarily of lean muscle meat, supplemented with a multivitamin/mineral supplement. In contrast, the free-ranging cheetahs on Namibian farmland have been shown, through scat analysis and direct observation, to consume a variety of common game species, consisting primarily of small to medium sized antelope and smaller quantities of warthogs, hares and birds (261). Wild cheetahs frequently consume the muscle tissue of the hind limbs of their prey, followed shortly by the abdominal and thoracic wall and contents. If left undisturbed, they will consume most of the carcass, leaving only the gastro-intestinal tract, skull and larger bones (59,61). Donkey meat is high in protein (22.8%) and low in fat (2.02%) (262), and thus comparable to a dressed springbok carcass which contains between 22.9 and 24.2% protein and 3.5 to 8.0% fat. Most African ungulates, however, have substantial intra-abdominal fat reserves, including, mesenteric, omental, perirenal and channel fat. Consumption of whole prey carcasses is therefore likely to result in an increased intake of dietary fat compared to captive diets that are limited to muscle meat and bone (263).

Furthermore, the fatty acid composition of the diet fed to the captive cheetahs is likely to differ with that of the diet consumed by free-ranging cheetahs. The high proportion of antelope in the free-ranging cheetah diet is expected to result in an increased proportional intake of saturated fat, since the unsaturated to saturated fat ratio in ruminants is substantially lower than in monogastric animals. Ruminant muscle tissue typically has an unsaturated to saturated fat ratio of between 0.11 and 0.15, while the ratio in horse meat ranges from 0.54 to 1.15 (256,264). A key feature of ruminant fatty acid metabolism is the hydrogenation of unsaturated components in the rumen. C18 PUFAs in the ruminant diet are converted to stearic acid prior to absorption and thus become the predominant fatty acid in their body tissues (265). Polyunsaturated fatty acids concentrations are therefore much lower in ruminant tissues than in those of monogastric animals. PUFAs make up only 4.78 and 5.88% of the total fatty acids of the muscle FAs in beef and lamb respectively, compared to 19.95 and 29.3% in pork and chicken (266).

The specific carcass components and organs consumed by captive versus free-ranging cheetahs may also explain the differences in serum fatty acids, as the internal fat depots, frequently consumed by free-ranging cheetahs, also have a higher proportion of saturated fat than either intramuscular or subcutaneous adipose tissue (267).

Although the total serum SFA concentrations were not significantly different between the captive and free-ranging cheetahs, the mean proportion of SFAs were higher at 86.1% of the total serum FAs, compared to only 61.7% in the captive animals, as shown in Table 6-4. This high proportion of SFA was also substantially higher than the 48.4% recorded in the plasma phospholipid FA fractions of 28 captive North American cheetahs by Bauer *et al* (76).

Heptadecanoic acid, otherwise known as margaric acid, is a saturated fatty acid with an uneven number of carbon atoms and is normally found at low concentrations in the adipose tissue and milk fat of ruminants. The mean serum concentration of this SFA was significantly higher in free-ranging cheetahs compared to those in captivity. Recently, the importance of this FA was demonstrated in another hypercarnivore, the bottlenose dolphin (*Tursiops truncates*) (268). Low serum heptadecanoic acid concentrations were associated with elevated serum concentrations of known markers of metabolic syndrome, including serum ferritin, glucose, insulin and triglycerides in the dolphins. A change in the fish species provided in the dolphin diet, with higher heptadecanoic acid content, resulted in a normalization of these markers. Two other FAs that correlated negatively with the markers of metabolic syndrome in dolphins were arachidonic acid and behenic acid. Both of these FA were also detected at significantly lower concentrations in the serum of captive cheetahs. Captive cheetahs are not known to suffer from metabolic syndrome, although it has been suggested that the glomerulosclerosis lesions, commonly found in these animals, may be caused by mild but chronic elevations in blood glucose concentrations (9). It is, nevertheless, interesting that this SFA, presumed to be non-essential in the diet, had such a dramatic metabolic effect in another obligatory carnivore. The importance of heptadecanoic acid in the diet of captive cheetahs therefore warrants further investigation.

Both the absolute concentrations of MUFAs and their proportions of the total serum FAs, were significantly lower in the free-ranging cheetahs. This was largely due to higher concentrations of palmitoleic acid and oleic acid in the serum of the captive animals. It is possible that this is simply a reflection of dietary intake of these fatty acids, since these two MUFAs make up about 34% of the FAs in donkey meat (264), whereas their combined proportion of muscular FAs in most game species is below 20% (269). However, both these MUFAs are also synthesised in the liver by stearoyl coenzyme-A desaturase (SCD) with palmitic and stearic acid as substrates. The regulation of SCD has been shown to have considerable physiologic importance, and alterations in SCD expression and regulation have been implicated in several metabolic diseases in humans and laboratory mice (270). The plasma ratio of C18:1 to C18:0 has been used as a “desaturase index” as a measure of SCD activity in humans (271). In SCD1 knockout mice, tissue levels of oleic and palmitoleic acid are reduced, while stearic acid and palmitic acid are increased. Such mice are also resistant to obesity and hepatic steatosis. Other than genetic mutations, elevated circulating insulin and glucose levels are known to increase SCD expression in the liver (272).

Serum insulin and glucose concentrations have not been adequately evaluated in non-anaesthetised captive or free-ranging cheetahs, but the levels would be expected to higher in the captive animals due to more regular feeding and a higher protein intake. Recently, evidence has emerged supporting a link between the metabolism of the sulphur amino acids (cysteine and methionine) and SCD expression (273). In Chapter 5, we documented a positive relationship between urine cysteine, as well as methionine, and the BMI values of captive cheetahs. Captive cheetahs with higher urine cysteine/methionine concentration, possibly due to higher dietary protein intake, could therefore have higher levels of hepatic SCD expression, resulting in elevated serum levels of oleic and palmitoleic acid concentrations. However, none of the serum fatty acid correlated significantly with BMIs in the captive cheetahs. In addition, the palmitic acid:palmitoleic acid and stearic acid:oleic acid ratios were calculated for the captive animals and they too did not show any significant correlation with the BMI values in captive cheetahs. The elevation of oleic acid and palmitoleic acid in captive cheetahs and the degree of SCD expression in these animals will require further investigation to determine the role, if any that they play in the chronic diseases suffered in captivity. It is however likely that mitochondrial β-oxidation is more active in the free-ranging cheetahs, since saturated fatty acid acyl-CoAs, but not monounsaturated fatty acid acy-CoAs, allosterically inhibit acetyl CoA carboxylase, reducing the intracellular levels of malonyl coA (274). Malonyl Co-A is required for the synthesis of fatty acids and also inhibits the mitochondrial carnityl palmityl shuttle system, which is the rate-limiting step in the importing of fatty acids into the mitochondrial for oxidation. Lower levels of SCD in the free-ranging cheetahs could therefore lead to lower levels of malonyl CoA, thus enhancing fatty acid oxidation.

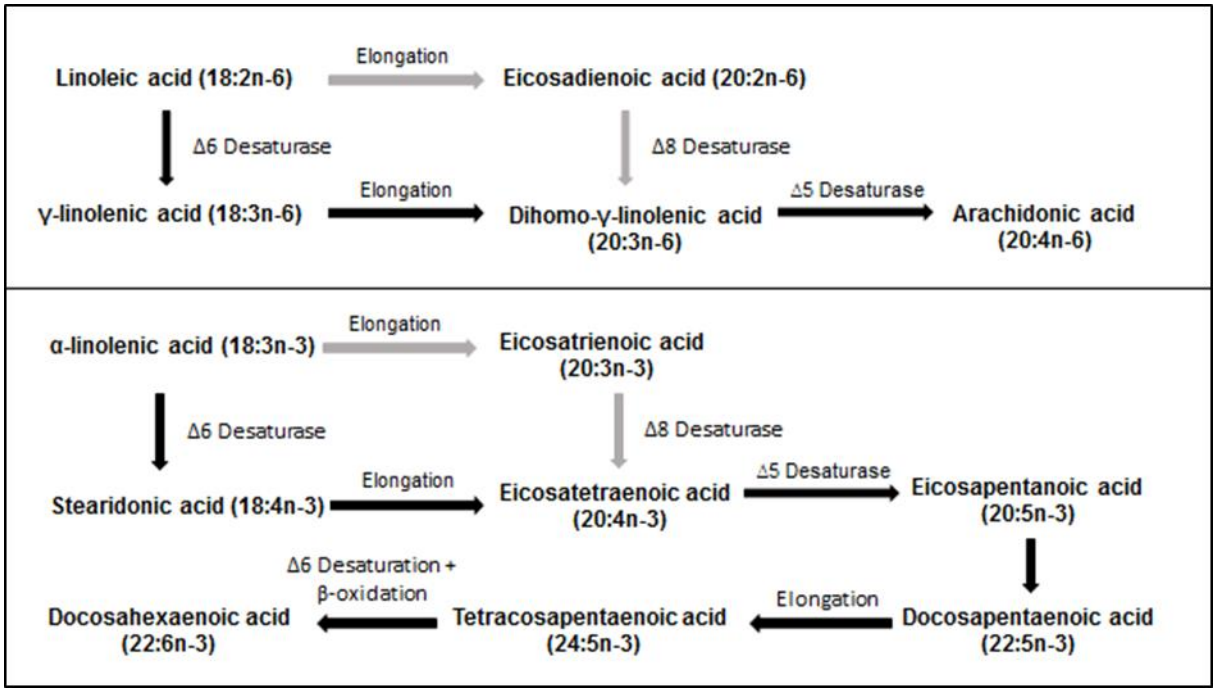
The difference between the serum PUFA concentrations in captive versus free-ranging cheetahs was particularly striking. Except for the higher levels of arachidonic acid in the free-ranging cheetahs, all the remaining PUFAs were detected at significantly lower concentrations in the free-ranging animals. This is surprising since PUFAs cannot be synthesised by mammals as they lack the ability to introduce double bonds beyond carbons 9 and 10 and therefore these fatty acids are considered be essential in the diet. Suspected deficiencies in PUFAs have been reported in the literature and appear to have responded to supplementation (258). In that report, two female cheetahs that had no oestrus cycle activity and suffered from poor coat condition, dull staring eyes and a scaly skin with sores around their anus and nostrils, were given a combination of supplements containing mostly omega-3 PUFAs, including γ-linolenic acid (C18:3ω6), EPA and DHA. Both animals showed significant improvement after only a month of supplementation and returned to oestrus, were bred successfully and produced a litter of cubs. Unfortunately no serum FA profiles were performed either before or after treatment. In our study, the free-ranging cheetahs had very low levels of the particularly the omega-3 FA series, with only low concentrations of EPA that were significantly lower than those of their captive counterparts. Most researchers and veterinarians, given the serum PUFA results, but blinded to the groupings of the

cheetahs in our study, would have diagnosed a deficiency in omega-3 essential fatty acids in the free-ranging cheetahs. Yet these animals were in good health, with none of the symptoms in the case report described above and clearly breeding successfully. Also surprising was the absence of either ALA or DHA in the serum of any of the cheetahs.

Several studies have been conducted on PUFA metabolism in domestic cats and these potentially provide some insight into the utilization of these FAs in a related species like the cheetah. When cats were fed LA as the only PUFA in the diet, only low plasma concentrations of AA were detected. When radio-labelled [1-14C] LA was administered, no desaturated fatty acid metabolites were detected. The administration of [2-14C] dihomo-γ-linolenic acid, resulted in a significant increase in AA levels. The authors concluded that cats have little ∆6-desaturase activity, but sufficient elongase and ∆5-desaturase activity (275). In subsequent studies, it was demonstrated that cats could use alternate pathways (as shown in Figure 6-6) to produce limited quantities of AA from LA (276). Studies have shown that, increases in dietary intake of ALA, result in elevated plasma levels of EPA but little or no change in tissue or plasma levels of DHA (277,278). In fact, both ALA and LA initially compete for active sites on ∆-6 desaturase and if either is present at high concentration in the diet, then there is little opportunity for tetracosapentaenoic acid (24:5ω-3) to bind to the same enzyme for the final conversion to DHA. High dietary concentrations of LA or ALA may therefore compete for very limited ∆-6 desaturase, preventing the formation of DHA, resulting a deficiency of this fatty acid if it is not already present in the diet.

The reason for the absence of DHA in the plasma of both captive and free-ranging cheetahs is potentially shown in an important study by Pawlosky *et al* in which they demonstrated that when deuterium labelled LA and ALA were administered to cats, their livers produced deuterium labelled long-chained FAs up to adrenic acid (22:4ω-6) and docosapentaenoic acid (22:5ω-3), but not DHA (279). Deuterium labelled DHA and several other long-chained fatty acids were however detected in the brain tissue of the cats, suggesting that the docosapentaenoic acid is transported from the liver to the brain, and probably the retina where it is then further metabolised to DHA and where it is critically required for normal function (280). In the absence of direct dietary supplementation of DHA, this fatty acid would thus be absent in the circulation and only synthesized in the brain and retina if sufficient ∆-6 desaturase were available.

Figure 6-6. Endogenous metabolic pathways of the ω-6 (above) and ω-3 series (below) of essential fatty acids. Black arrows indicate standard pathways, while grey arrows indicate alternate pathways.



It therefore appears that cheetahs have adapted to a diet low in PUFAs, with the various essential FAs provided within narrow limits for optimal health. A full complement of all the essential PUFAs could be provided in the diet of captive cheetahs to ensure adequate levels are provided, but PUFAs are prone to peroxidation and this might increase the risk of steatitis in these animals if additional vitamin E is not also provided (281). Wild cheetahs only consume freshly killed prey and would therefore rarely be exposed to peroxidized fatty acids. The feeding of stored meat products with a high polyunsaturated fatty acid content therefore may pose health risks to captive cheetahs, since unsupplemented meat is unlikely to contain sufficient vitamin E reserves (282).

The increased proportional intake of dietary fat, decrease in feeding frequency and increased physical activity in free-ranging compared to captive cheetahs are all predicted to result in enhanced mitochondrial FA oxidation through the lowering of circulating glucose concentrations and insulin:glucagon ratios (283). During fasting/refeeding cycles and increased levels of exercise, tissue PUFA concentrations have been shown to deplete rapidly in both humans and rats (284,285). These studies show that most PUFAs, including α-linolenic acid (ALA) and linoleic acid (LA), are preferentially oxidized in periods of exercise or fasting. During refeeding, SFAs and monounsaturated fatty acids (MUFAs), such as palmitic acid and oleic acid, are also more rapidly replaced than any of the PUFAs. Similarly, the concentrations of most plasma PUFAs and MUFAs have been shown to be significantly lower in rats fed a high fat ketogenic diet than in controls

(286). An increase in FA oxidation in free-ranging cheetahs is therefore likely to also skew their serum FA profiles toward lower proportional serum concentrations of PUFAs and MUFAs relative to SFA.

The higher serum concentrations of pristanic and phytanic acids in the free-ranging cheetahs provide further evidence of their greater consumption of ruminant prey. Phytanic acid (3,7,11,15-teramethylhexadecanoic acid) is produced from phytol, the side chain of chlorophyll, by the micro-organisms present in the ruminant gastrointestinal tract (287). The β-methyl group in phytanic acid prevents its degradation by β-oxidation since the production of a required 3-ketoacyl CoA intermediate is not possible. Phytanic acid therefore undergoes a single cycle of peroxisomal α-oxidation to form pristanic acid. Pristanic acid is then degraded through 3 cycles of perioxisomal β-oxidation before being esterified to carnitine and transported to the mitochondria for further oxidation. Certain genetic defects related to perioxisomal fatty acid oxidation in humans have been shown to lead to very high serum levels of phytanic acid (288), but little is known about the potential positive or negative effects of these branched-chain fatty acids when they are consumed in the diet.

In the captive cheetahs, a weak negative association was found between the age of the cheetahs and serum eicosadienoic acid and arachidic acid concentrations (Figure 6-3). Pristanic acid also declined relative to and the ratio of pristanic acid to phytanic acid showed a similar decline relative to age (Figure 6-5). It is unlikely that that these associations would be due to variation in dietary intake of these FA relative to age, since all the captive cheetahs are fed a similar diet. In the case of pristanic acid, the decline relative to age could be due to a decrease in peroxisomal oxidation relative to age, but it is not clear how such decline would occur. The ratio of pristanic acid to phytanic acid also declined significantly relative to age, but phytanic acid did not accumulate in the serum of older captive cheetahs. In the free-ranging cheetahs, a few FAs increased relative to age (arachidonic acid, stearic acid and heptadecanoic acid) while hypogeic acid decrease relative to age. The reason for these associations is not known, but it is possible that as the free-ranging cheetahs matured, their prey preferences changed, resulting in altered fatty acid intake.

Most saturated very long-chained fatty acids are components of sphingolipids. The C24 sphingomyelin, which incorporates lignoceric acid, is particularly abundant in liver and kidney tissue (289) and therefore higher serum concentrations of lignoceric and behenic acid in the free-ranging cheetahs may simply reflect their higher dietary intake of these organ meats.

As with the fatty acids, the carnitine and acylcarnitine concentrations in the serum of captive and free-ranging cheetahs differed significantly in all but a few cases, once again reflecting major differences in fatty acid metabolism between these two sampled populations. The serum carnitine concentrations were significantly higher in the captive animals, while most of the acylcarnitines

were detected more frequently or at higher concentrations in the free-ranging cheetahs. In adult humans, the dietary intake of carnitine correlates with plasma carnitine concentrations (290). Approximately 75% of total body carnitine originates from food sources rich in carnitine, or endogenous synthesis from dietary methionine and lysine. Carnitine is particularly abundant in red muscle meat and the higher concentration of serum carnitine concentrations in captive cheetahs is therefore no surprise.

Although most acylcarnitines are formed during fatty acid oxidation, they can be formed from almost any coenzyme A ester. For example, other intermediates that can yield acylcarnitines include amino acid degradation products from lysine, tryptophan, valine, leucine and isoleucine (e.g. C3 and C5-carnitines); ketone bodies (C4-3OH-carnitine) and from glucose (C2-carnitine) (291). The comparable serum C5-carnitine species in captive and free-ranging cheetahs could therefore reflect similar amino acid metabolism in these two groups. During starvation or the intake of a high-fat diet, the proportion of carnitine that is acetylated in the liver and kidney increases and greater proportions of both short and long-chain acylcarnitines are released into the circulation (292). The higher concentrations of the majority of the acylcarnitines in free-ranging cheetahs suggest, in line with other findings, that they are consuming more fat in their diet and relying to a greater extent on fatty acid oxidation for energy production.

Serum concentrations of the short chain acetylcarnitine (C2-carnitine) were significantly higher in the free-ranging cheetahs. Recent studies have shown beneficial effects of this acylcarnitine, particularly in the central nervous system (reviewed in (292)). It is derived from the acetylation of carnitine in the mitochondria, enabling the function of coenzyme A and facilitating the elimination of oxidative products. Acetylcarnitine also provides acetyl groups for acetylcholine synthesis, has cholinergic effects and enhances the balance of energy processes. Acetylcarnitine is present at relatively high concentrations in the brain, particularly in the hypothalamus. It readily crosses the blood-brain barrier, where it appears to have neuroprotective effects by improving mitochondrial function through various mechanisms. It also provides high-energy acyl groups to various metabolic pathways, improving overall energy status of the brain. Evidence also suggests that acetylcarnitine plays a role in the elongation and desaturation of the n-3 polyunsaturated fatty acids to form docosahexaenoic acid (DHA) in the mitochondria.

6.5 Conclusion

In this study, the total serum FA profiles of 43 apparently healthy free-ranging cheetahs in Namibia were documented. The unusual diseases suffered by captive cheetahs are rarely reported in free-ranging cheetahs (18). Thus, the FA profiles of free-ranging cheetahs provide a reasonable set of reference values against which the profiles of captive individuals can be assessed. The potential links between the diseases suffered by captive cheetahs and the FA composition of their

diet, however, still remain unclear. Future studies will need to focus on evaluating the associations between the incidence and/or severity of disease and the various serum FAs, as well as the clinical effects of dietary manipulations and/or FA supplementation. The relative effects of extended feeding intervals and increased physical activity on serum FAs in captive cheetahs will also need to be assessed. Free-ranging cheetahs rarely scavenge and consume most of their prey within a few hours after a kill. Fats consumed under these conditions have little time to become rancid and the higher intake of the oxidatively more stable SFAs would result in a reduced need for extensive antioxidant mechanisms to deal with peroxidised FAs in this species. The extent and impact of dietary FA peroxidation should therefore also be assessed in future studies.

A limitation of the study was the lack of control over the fasting period prior to sampling in the free-ranging cheetahs. Although no bait was used in their capture traps, it is possible that some of the cheetahs may have fed within a few hours before immobilization and sampling. During the postprandial period, the non-esterified fatty acids (NEFAs) generally decline in response to rising insulin levels, while the triacylglycerol lipid fractions increase slightly or remain stable in healthy individuals (293). Since the NEFA fraction makes up only approximately 6% of the total serum FAs (294), the postprandial variation in total serum FAs is likely to be small. Nevertheless, it would be valuable to assess the changes in serum FAs during the postprandial period in healthy captive cheetahs fed on different diets.

An additional potential limitation in our study was the differences in serum separation and sample storage times prior to analysis in the captive versus the free-ranging cheetahs. Although circulating NEFA fractions potentially increase in blood and plasma after 48 hours at 4°C (295), storage of various plasma lipid classes, including triglyceride, cholesterol ester, phospholipid and NEFA fractions at -20°C for a year, was shown to have minimal effect on stability even without the use of nitrogen storage (296). In a recent review, Metherel and Stark concluded that plasma/serum storage at 4°C for less than 6 days, -20°C for one to three years and -80°C for up to 10 years would not likely result in significant changes in PUFA concentrations. The effects of delayed blood cell separation on total serum or plasma FA concentrations have not to our knowledge been evaluated. However, a delay of 24 hours before separation in samples kept at 4°C, resulted in an increase in NEFA concentrations of between 0% and 8% in plasma (297,298) and 10% in serum (297). These increases are most like due to the action of lipolytic enzymes which result in a FA shift from the bound fractions to free FA pool. Such changes are not likely to affect the combined/total plasma or serum FA concentrations and therefore the differences in sample handling, prior to storage, would be expected to have a minimal effect on our results. The impact of different sample handling procedures on total serum or plasma FAs under field conditions should nevertheless be investigated in future studies.

CHAPTER 7
GENERAL DISCUSSION

In a captive environment, cheetahs experience a high incidence of several chronic non-infectious diseases that are rarely seen their free-ranging counterparts or in other captive felids (10,11,18). Although the longevity of this species in captivity has improved over the last few decades, only a few facilities have managed to breed cheetahs successfully (7,25). There has been much speculation in the last 30 years about the reasons for their ill health and poor reproductive performance, but causal factors and disease mechanisms have to date not been clearly identified. In the 1980s, the cheetah was put forward as a prime example of a species suffering from inbreeding depression and genetic impoverishment (38) and it was suggested that much of their poor health and relative infertility was due to this low heterozygosity. This idea was, however, challenged when it became apparent that free-ranging cheetahs, with the same level of genetic diversity as those in captivity, had high reproductive rates and very low levels of disease (3,18,32). The focus then shifted to the vulnerability of cheetahs to stress in captivity and Terio *et al* (16) provided convincing evidence, through faecal cortisol evaluations as well as post mortem adrenal measurements, that cheetahs in captivity were significantly more stressed than free-living individuals. Similar results were however not demonstrated in all captive populations (32) and it became clear that stress alone could not account for the high levels of disease in almost all captive facilities. In recent years, the role of nutrition in the diseases of captive cheetahs has received more attention (63,79,80), These epidemiological studies seem to indicate a link between the macronutrient components in cheetah diets and gastro-intestinal health, but to date no clear links have been established between any dietary components and the incidence or severity of any of the clinical conditions seen in this species.

Disease investigations in cheetahs are constrained by several factors including the chronic nature of the diseases in question, high variability in diets and nutritional supplements provided, the small numbers of animals at individual facilities, the difficulty in obtaining samples from non-anaesthetised animals and the lack of baseline physiological data for the species. Furthermore, many of the diseases that afflict captive cheetahs are chronic and only diagnosed at post mortem. The diagnosis of mild to moderate glomerulosclerosis, for example, would require the collection of invasive renal biopsies and the standard procedure for the evaluation of lymphoplasmacytic gastritis necessitates gastric endoscopy and the collection of several gastric mucosal biopsies (22).

Using a new systems biology approach, our aim in this study was therefore firstly, to broaden our baseline knowledge of the cheetah metabolome in order to provide a sound platform for future research in this species and secondly, to generate new hypotheses which could in turn lead to

more targeted studies. Research of this nature had not been conducted before in captive wild animals, and the methods used in humans, domestic species and laboratory animals had to be adapted for the cheetah.

Intrinsic factors, such as age, sex and body composition, could significantly affect the metabolome of a species. The potential impact of these variables on metabolite concentrations therefore had to be evaluated. Various body condition scoring systems have been created for both domestic and wild carnivores. These scoring systems suffer from a high degree of inter-observer variability. In order to develop a more objective way of assessing body condition in cheetahs, we developed a body mass indexing system for cheetahs based on body measurements in two dimensions (shoulder height and body length). Both measurements are not influenced by either lean muscle mass or body fat percentage and thus provide a good indication of body size, independent of changes in body condition. When combined with body weight, the calculated value should provide a good approximation of body condition, especially in an animal like the cheetah where body conformation is relatively uniform. The cheetah BMI values, however, do not distinguish between muscle mass and fat reserves and subtle shifts in these may therefore not be accurately reflected. Nevertheless, under field conditions, the body mass indexing system outlined in this study provides the most objective assessment of body condition available in this species. As additional morphometric data is collected from both captive and free-ranging cheetahs, normal reference ranges for BMI values can be established for captive cheetahs. The relative impact of BMI-defined obesity or emaciation on the metabolome can also be assessed in future cheetah studies.

The collection of 24-hour urine samples are, for obvious reasons, completely impractical in a wild animal like the cheetah and therefore metabolites have to be measure in spot urine samples obtained at the time of immobilisation. Urine creatinine concentrations have traditionally been used to correct or adjust the concentrations of other metabolites, drugs or toxins. This is based on the assumption that creatinine is produced at a fairly constant rate and is excreted unchanged in the kidneys. Creatinine production is thought to be largely reflective of muscle mass, varying to some extent with age, sex physical exercise and dietary intake in humans (129,130). In this study we documented dramatic variation in the creatinine excretion of cheetahs. This variation was shown to be largely influenced by unknown age-related factors and not significantly affected by the sex or muscle mass of individuals. We therefore argue that in cheetahs, the correction of spot urine metabolites relative to urine creatinine concentrations, will more than likely lead to an overestimation of these compounds in younger and older animals. Urine specific gravity, has been shown to be a reliable alternative correction factor for the correction of urine metabolites in humans (136,137). In healthy carnivores, where urine is relatively free of glucose, haemoglobin, bilirubin and other larger molecules, urine SG would be expected to provide a good approximation of urine osmolality. The cheetahs in our study received a diet with fairly consistent levels of protein

and where fasted for the same period of time prior to sample collection. We would therefore expect urine production to be fairly constant. Urine specific gravity is likely to provide a good estimate of urine dilution due to individual differences in water consumption in cheetahs, and should therefore provide a more accurate means of correcting the concentrations of metabolites in spot urine samples. It would be ideal to evaluate the accuracy of this method by comparing it to values obtained from 24-hour samples, but obtaining such samples is simply not practical in an animal like the cheetah.

Other than for its use in correcting spot urine metabolite concentrations, this study raises several other important questions about the production and metabolism of creatinine in the cheetah. Creatine provides an important source of energy through conversion to its high-energy phosphorylated form, creatine phosphate. Large creatine phosphate reserves are theoretically an important source of energy in the muscle metabolism of the world's fastest land mammal, allowing extreme speed, over short distances. Large pools of creatine phosphate are available in fast-twitch muscle fibres for rapid regeneration of ATP, hydrolysed during intense exercise (299). Creatine is however not only important for muscle cell energetics, but is also required for energy regulation in the brain and has antioxidant functions (300).

Since the captive cheetahs in this study all received a very similar diet, the decline in creatinine excretion in older cheetahs is not likely to reflect differences in dietary intake. These animals were fed a diet high in muscle meat, which is one of the nutritional sources with the highest creatine content. The variation therefore must arise from either reduced intestinal absorption of creatine and creatinine or arise due to a decline in *de novo* creatine synthesis. Creatine in the diet normally has a bioavailabitity of around 80% (299). In the small intestine, creatine is actively transported across the brush border membrane by a Na^+; Cl^- cotransporter (301). The impact of chronic gastrointestinal inflammation on the absorption of creatine in cheetahs has not been assessed and may be worth investigating. *De novo* synthesis of creatine takes place in the kidneys, liver and pancreas via two enzymatic reactions that utilise arginine, glycine and S-adenosylmethionine (SAM) as substrates. As argued in Chapter 4, the high demand for the glycine conjugation of phenolic compounds in captive cheetahs, could reduce the pool of glycine available for creatine synthesis. The synthesis of N^1,N^5-Dimethylpentane-1,5-diamine, the cadaverine derivative, detected at high concentrations in the urine of captive cheetahs, requires two sequential methylation reactions that utilise SAM. As creatine production already requires approximately 40% of all the methyl groups provided by SAM (299), the additional demand for methyl groups on captive cheetahs could lead to a deficiency of methyl donors. This deficiency could be further exacerbated by a vitamin B12 or folate deficiency, since both are required in transmethylation reactions. Why this might get progressively worse with age in captive cheetahs, is however, not

clear. The decline in creatinine excretion, relative age in captive cheetahs may reflect more complex metabolic abnormalities and clearly warrants further investigation.

Two novel compounds were detected at very high mean concentrations in the urine of the captive cheetahs. As discussed earlier, one of these has been tentatively identified as N^1,N^5-Dimethylpentane-1,5-diamine, a derivative of the diamine cadaverine, which in turn is formed by the bacterial decarboxylation of the amino acid lysine. Work is currently underway to identify the second compound, presently labelled as Unknown 362. Besides these two metabolites, several phenolic compounds derived from the enteric bacterial metabolism of the aromatic amino acids, phenylalanine and tyrosine, were also detected at high concentrations in the urine of captive cheetahs. These results suggest that large quantities of these amino acids escape intestinal absorption and are available for colonic bacterial degradation. This surplus of amino acids is, more than likely, the result of the high muscle meat intake leading to excessive amounts of protein being available for digestion. Bechert *et al* showed that supplemented meat diets fed to cheetahs had a protein to fat ratio of 6.6:1 (302), while in the whole prey eaten by free-ranging cheetahs, this ratio is only around 3:1 (263). It is likely that such alterations in the macronutrient composition of the cheetah diet would in turn dramatically alter their intestinal microbiome. The intestinal bacterial communities of two captive cheetahs analysed with 16S rRNA gene clone libraries (303), were dramatically different from those of free-ranging cheetahs (304). The captive cheetahs had very high proportions of Firmicutes (94.7%) compared to the free-ranging cheetahs (56.2%). The Firmicute phylum includes Clostridia, a class of bacteria that are known for their fermentation of aromatic amino acids (305). The proportion of Fusobacteria in these two studies also differed dramatically, making up only 0.6% in the captive cheetahs versus 18.1% in the free-ranging animals. The intestinal microbiome is known to primarily be modified by the macronutrient composition of the diet (306) and plays an import part in the metabolism of the host (307). In our study, at least 13 of the 30 most abundant urinary organic acids potentially originate from intestinal bacterial metabolism. It is therefore quite possible that differences in the macronutrient content of captive cheetah diet may alter intestinal bacterial communities, changing key aspects of their metabolism. Evidence of this is shown by the relationship between the urine phenolic compounds and the end-stage metabolites of dopamine and noradrenaline (VMA and HVA) in our study. In Chapter 4, we suggest new potential pathophysiological mechanism for gastrointestinal disease and glomerulosclerosis in captive cheetahs. Both proposed mechanisms relate to the inhibition of key enzymes responsible for the production of dopamine and noradrenaline, resulting in neuro-endocrine dysregulation. Further research is however required to establish clearer links between these metabolite changes and the incidence and/or severity of these diseases. Research of this nature has already been undertaken to evaluate the relationship between the gastritis scores (as determined by gastric biopsies) and the relevant urine organic acids discussed in the present

study. Carefully planned diet-trial studies in captive cheetahs would also be very helpful to further test these hypotheses.

Unfortunately, suitable urine samples were only obtained from two free-ranging cheetahs in our study. Although a few additional urine samples were also collected from free-ranging cheetahs by other researchers and veterinarians, these animals were cage trapped prior to immobilization and the influence of food consumption and stress before sampling could not be controlled for. Nevertheless, the results from these two animals do provide some valuable insights. Statistical analyses (PCA and PLS-DA) placed the profiles of these two individuals as distinct, clustered outliers to those of the larger group of captive cheetahs. The urine organic acid metabolites that differed the most between the captive and free-ranging cheetahs have their origins in fatty acid metabolism. In all cases the fatty acid metabolites were detected at higher concentrations in the free-ranging cheetahs than in the captive animals. This seems to confirm that these two animals consumed a diet higher in fat. Both these cheetahs also had very low urine concentrations of the unidentified compound (Unknown 362) and the newly identified N^1,N^5-dimethylpentane-1,5-diamine. The impact of these two compounds on the health of captive cheetahs is currently unknown, and given the high concentrations detected in the urine of the captive animals, further research on these two metabolites should be prioritised. Additional urine samples, obtained from free-ranging cheetahs would also be very valuable to evaluate against the findings of this study.

The serum and urine amino acid data obtained from captive cheetahs in this study provided fewer clear insights into the health of these animals. Serum amino acids are known to be tightly regulated except in the postprandial period and during extended fasting (221). Amino acids are also metabolised through multiple pathways and therefore subtle changes in their serum or urine concentrations are therefore more difficult to interpret. The results however provide important baseline data. Additional information on the changes of serum and urine amino acid concentrations in the postprandial period would provide additional insights into the metabolism of amino acids in this species. The study of certain amino acids and dipeptides related to the metabolism of collagen, such as proline, hydroxyproline, and prolylproline, may also be valuable in young growing cheetahs to develop a clearer understanding of their use in diagnosing subclinical metabolic bone disease.

The comparison of serum fatty acid and acylcarnitine profiles between captive and free-ranging cheetahs provides some unique insights on the differences in dietary fatty acid composition and/or metabolism in these two groups. To our knowledge there are no other studies that assess the serum or plasma fatty acid profiles of a free-ranging felid species. The non-captive cheetahs sampled in this study were trapped on commercial and communal farmland, where they would have access to a wide range of natural prey species. These animals therefore serve as valuable controls against which the results from captive animals can be compared.

The serum fatty acid, carnitine and acylcarnitine profiles of captive cheetahs contrasted dramatically with those in the free-ranging individuals. The proportions of saturated, mono-unsaturated and polyunsaturated fatty acids in the serum of captive and free-ranging cheetahs were significantly different, with saturated fatty acids making up 86.1%, monosaturated fatty acids 5.2% and polyunsaturated fatty acids 8.7%. Polyunsaturated fats are often thought to be healthier than saturated fats, but the higher the number of double bonds, and the longer the carbon chain, the more prone fatty acids are to oxidation. Pansteatitis, an inflammatory condition of adipose tissue has been documented in domestic cats that are fed a diet high in unsaturated fatty acids, especially where the levels of vitamin E provided are inadequate (253). It appears that cheetahs may have adapted to a diet low in polyunsaturated fatty acids, with the various essential fatty acids provided within narrow limits for optimal health. They potentially maintain a low polyunsaturated fatty acid intake by largely consuming small ruminants, which have a high saturated to polyunsaturated ratio. In contrast, captive cheetahs are frequently fed carcass meat and fat from monogastric animals, with higher levels of polyunsaturated fatty acids, such as chickens, donkeys and horses. The high polyunsaturated diets may not necessarily contain sufficient eicosapentaenoic acid and docosahexaenoic acid, and high levels of linoleic acid or α-linolenic acid may compete for very limited ∆-6 desaturase, preventing the formation of the longer chain essential fatty acids. Besides providing a valuable source of energy, fatty acids also perform other vital functions in the body, including hormone production, cellular signalling as well providing structural components for biological membranes. The consumption of diets with abnormal fatty acid content and composition could therefore have a profound effect on the health of these animals.

In conclusion, the evaluation of the cheetah metabolome has provided a sound platform for future research and has highlighted several potential areas in which the unnatural diets fed to captive cheetahs potential cause derangements in their metabolism, ultimately leading to chronic disease. New hypotheses are put forward in this thesis providing new directions for future cheetah research, hopefully improving the health and wellbeing of these magnificent animals.

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APPENDIX 1

PLOS ONE

RESEARCH ARTICLE

Comparative Serum Fatty Acid Profiles of Captive and Free-Ranging Cheetahs (*Acinonyx jubatus*) in Namibia

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Abstract

Cheetahs (*Acinonyx jubatus*) are highly specialised large felids, currently listed as vulnerable on the IUCN red data list. In captivity, they are known to suffer from a range of chronic non-infectious diseases. Although low heterozygosity and the stress of captivity have been suggested as possible causal factors, recent studies have started to focus on the contribution of potential dietary factors in the pathogenesis of these diseases. Fatty acids are an important component of the diet, not only providing a source of metabolisable energy, but serving other important functions in hormone production, cellular signalling as well as providing structural components in biological membranes. To develop a better understanding of lipid metabolism in cheetahs, we compared the total serum fatty acid profiles of 35 captive cheetahs to those of 43 free-ranging individuals in Namibia using gas chromatography-mass spectrometry. The unsaturated fatty acid concentrations differed most remarkably between the groups, with all of the polyunsaturated and monounsaturated fatty acids, except arachidonic acid and hypogeic acid, detected at significantly lower concentrations in the serum of the free-ranging animals. The influence of age and sex on the individual fatty acid concentrations was less notable. This study represents the first evaluation of the serum fatty acids of free-ranging cheetahs, providing critical information on the normal fatty acid profiles of free-living, healthy individuals of this species. The results raise several important questions about the potential impact of dietary fatty acid composition on the health of cheetahs in captivity.

Introduction

Cheetahs (*Acinonyx jubatus*) are kept in many facilities worldwide. Despite obvious improvements in husbandry since cheetahs were first kept in captivity, they still suffer from a range of

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Gas chromatography-mass spectrometry profiles of urinary organic acids in healthy captive cheetahs (*Acinonyx jubatus*)

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ABSTRACT

In captivity, cheetahs (*Acinonyx jubatus*) frequently suffer from several unusual chronic diseases that rarely occur in their free-ranging counterparts. In order to develop a better understanding of their metabolism and health we documented the urine organic acids of 41 apparently healthy captive cheetahs, in an untargeted metabolomic study, using gas chromatography–mass spectrometry. A total of 339 organic acids were detected and annotated. Phenolic compounds, thought to be produced by the anaerobic fermentation of aromatic amino acids in the distal colon, as well as their corresponding glycine conjugates, were present in high concentrations. The most abundant organic acids in the cheetahs' urine were an as yet unidentified compound and a novel cadaverine metabolite, tentatively identified as N^1,N^5-dimethylpentane-1,5-diamine. Pantothenic acid and citramalic acid concentrations correlated negatively with age, while glutaric acid concentrations correlated positively with age, suggesting possible dysregulation of coenzyme A metabolism in older cheetahs. This study provides a baseline of urine organic acid reference values in captive cheetahs and suggests important avenues for future research in this species.

1. Introduction

The cheetah (*Acinonyx jubatus*) is a highly specialised large felid, listed as vulnerable on the International Union for Conservation of Nature (IUCN) red list and the last surviving member of the Acinonyx genus [1]. Considered to be the world's fastest land mammal [2], this species is quite unique it terms of its morphological features that have been described to be somewhat intermediate between that of a wolf and other felids [3].

In captivity, cheetahs are known to suffer from a number of unusual diseases not typically seen in other captive felids. These include lympho-plasmacytic gastritis [4] with associated renal amyloidosis [5,6], glomerulosclerosis [7,8], hepatic veno-occlusive disease [9], splenic myelolipomas [10], cardiac fibrosis, adrenal cortical hyperplasia with lymphocyctic depletion of the spleen [7], as well as several idiopathic neurological disorders [11,12]. Gastritis and renal disease eventually affect the majority of cheetahs in captivity and are considered to be the primary cause of morbidity and mortality in adults [7]. In contrast, these diseases are rarely detected in free-ranging individuals [13]. Stress, lack of exercise, low genetic variability and the provision of unnatural diets in captive facilities have been proposed as potential causal factors, but to date convincing pathophysiological explanations for these diseases have been lacking or unsatisfactory.

Urine is valuable from an analytical point of view as it contains a large number of metabolites, produced by homeostatic mechanism within an organism, that are likely to remain relatively unaltered if collected soon after capture and immobilization. Urinary organic acids are a broad class of water-soluble end-products and intermediates of a wide range of metabolic pathways, including the metabolism of amino acids, carbohydrates, fatty acids, ketones and biogenic amines. The quantification of urinary organic acids has primarily been used in the detection and management of inborn errors of metabolism [14]. Changes in the urinary excretion of organic acids have, however, also provided valuable information for the early diagnosis of metabolic [15,16] and neurological disorders

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