Metabolic effects of glycine supplementation in captive cheetahs (*Acinonyx jubatus*)

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DECLARATION

I, *Kathryn van Boom*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature:

Date: 5 July 2023

DEDICATION

This is dedicated to my mom, who saw no part of this journey,
but who showed me how to dream and how to work hard to achieve them.

PREFACE

Study motivation

Cheetahs are a vulnerable species and their management and conservation is of global importance. In captivity, cheetahs are prone to a wide range of diseases (such as gastritis and renal disease) that are not seen in their free-roaming counterparts (Munson et al., 1999). It is believed that certain factors linked to captivity, like diet, may play a role in the high prevalence of these diseases. In the wild, cheetahs would eat whole carcass diets rich in collagen due to the bones, skin and connective tissue (Depauw et al., 2013). However, these diets are not always feasible in captive settings which results in most captive facilities predominantly feeding their cheetahs a variety of raw muscle meat, such as chicken, horse and beef (Whitehouse-Tedd et al., 2015). Glycine is a simple, inexpensive, highly palatable amino acid with a range of physiological functions in mammals, most notably its role on collagen biosynthesis (Wang et al., 2013). Anecdotal evidence suggests that dietary glycine supplementation may be beneficial for cheetahs in captivity – however, the metabolic effect of glycine supplementation in cheetahs has not yet been investigated. Therefore, the primary focus of this study was to determine the effect of glycine supplementation on the metabolism of the cheetah through NMR techniques which would provide a holistic and clear picture of the many metabolic pathways involved in their metabolism. This study would provide important information on the effects of glycine which may also have implications in other felid species and wildlife.

Summary of samples collected

A total of 30 blood, urine, *Vastus lateralis* skeletal muscle biopsies and 270 gastric biopsies (9 per cheetah per sample collection) were collected from 10 captive cheetahs during 3 different sample collection periods in 2020. Samples were collected before, during and at completion of the diet intervention. Cheetahs were housed at Cango Wildlife Ranch in Oudtshoorn, Western Cape. Only the results obtained from the urine and blood are reported in this thesis. Ethical approval for this study was obtained from the Animal Research Ethics Committee of the Faculty of Veterinary Science, University of Pretoria and the Animal Research Ethics Committee of the University of the Western Cape.

Structure of the thesis

Chapter one provides a literature review discussing various aspects including the diseases and diets of captive cheetahs, the physiological effects of glycine and the benefits of a metabolomics approach. Chapter two describes the methodology of the study from the study design and sample collection to the data and statistical analysis. Chapter three discusses the body measurement, serum biochemistry and haematology results. Chapter four and five discuss the urine and serum

metabolites, respectively, that were identified with NMR. Lastly, chapter six provides a general discussion of the findings and the conclusion.

Planned outcomes of the study

One article entitled “A cross-over dietary intervention in captive cheetahs (*Acinonyx jubatus*) – investigating the effects of glycine supplementation on blood parameters” has been submitted and is under review by Zoo Bioogy (submitted February 2023 and re-submitted May 2023).

There are two planned publications entitled “The effect of glycine on urine and serum metabolomics in captive cheetahs” and “The effect of glycine on skeletal muscle properties in captive cheetahs” for 2023 with the latter publication utilising additional data not included in this thesis as it was out of the scope.

Author and study leader contributions

The study was conceived by Prof Adrian Tordiffe (co-supervisor) and designed by myself, Prof Tordiffe and Prof Tertius Kohn (supervisor). Funding for the study was obtained by Prof Kohn (NRF) and myself (SAVF). Prof Tordiffe immobilised all of the cheetahs and performed sample collection during immobilisation at Cango Wildlife Ranch, Oudtshoorn. Sample analysis was performed by technicians in the Clinical Pathology laboratory, Department of Companion Animal Clinical Studies, University of Pretoria and the Centre for Human Metabolomics, North West University. I planned the logistics of the study, obtained ethical approval, managed the feeding intervention, assisted in the sample and data collection and performed all data and statistical analyses pertaining to the study. I also wrote this thesis, the submitted journal manuscript and I will write the planned future manuscripts.

Acknowledgements

There are many people I need to thank for their important roles in the completion of this study. Firstly, I would like to thank my supervisor, Prof Tertius Kohn, who has been my mentor since we started working together in 2016. Thank you for all your support and guidance throughout this journey and for showing me what it means to be a thoughtful and passionate researcher. To think that we first met when I was in my second year of my undergraduate degree, and to come full circle, nearly 10 years later, is an absolute privilege. It is a testament to your character as a leader and mentor – I am exceedingly grateful that you have travelled this journey alongside me.

Thank you to my co-supervisor, Prof Adrian Tordiffe, who was instrumental in getting this project off the ground and whose passion for cheetahs and their wellbeing has been a driving force in this field of research. I am grateful for the opportunity to have worked with you and I have learnt so

much in the process. Thank you for your expertise and for your assistance with the ethics process, sample collection, data analysis and interpretation and reviewing of this thesis.

Thank you to the staff at Cango Wildlife Ranch in Oudtshoorn, Western Cape for their involvement in this study particularly during the feeding intervention and sample collection phase. I would like to specifically mention Narinda Beukes, Craig Gouws, Tamblyn Williams and Riëtte Koortzen who went above and beyond to ensure the success of this project. It was a pleasure to work with you all.

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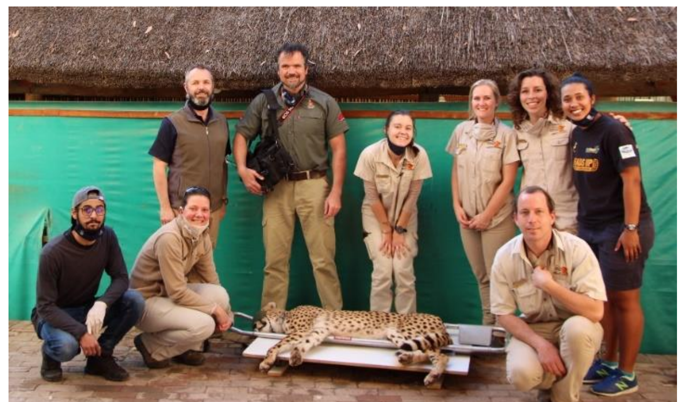
Thank you to my fellow students, Dean Herbig and Channen Long, for providing assistance in the data and statistical analysis. As well as Luqmaan Adamson for assisting during the sample collection period. Thank you to Kerryn Mac Dermott for reviewing parts of this thesis and for your encouragement throughout. I would also like to extend my thanks to Madelyn de Wet and Leonie Johnson at the University of Pretoria for all their administrative assistance.

My deepest thanks and appreciation goes to my dad, Garth van Boom. You have been my biggest supporter and fan since day one. Thank you for always being there for me on the good days and bad days and for always taking an interest in my work. Your heart for people has shown me what it means to be a kind, compassionate and loving human being which I hope to emulate my entire life. I love you.

Lastly, I would like to acknowledge and thank God for creating this beautiful world and all the majestic animals that roam it. By your grace, you have guided me along this path and given me the opportunity to explore and understand your creation.



LIST OF KEY ABBREVIATIONS

1,3-DAP	1,3-diaminopropane
a.u	arbitrary unit
AABA	α-aminobutyric acid
Alb	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
ASICS	Automatic Statistical Identification in Complex Spectra
ATP	adenosine triphosphate
BCFA	branched chain fatty acid
BMI	body mass index
CMP	cytidine monophosphate
CoA	coenzyme A
CSF	cerebrospinal fluid
dH20	distilled water
DHA	dehydroascorbic acid
DHP	dihydropyrimidinase
DMA	dimethylamine
$DMSO_2$	dimethyl sulphone
DNA	deoxyribonucleic acid
FDR	false discovery rate
g	gram
g	gravity
G-6-P	glucose-6-Phosphate
GABA	γ-aminobutyric acid
GC-MS	gas chromatography mass spectrometry
GCS	glycine cleavage system
GMP	guanosine monophosphate
HPLC	high performance liquid chromatography
Hz	Hertz
IQR	interquartile range
IU	International unit
IUCN	International Union of Conservation of Nature
K	Kelvin
kDa	kilodalton
kg	kilogram
L	litre
LC-MS	liquid chromatography mass spectrometry
m	metre
M	molar
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration

MCM	methylmalonyl-CoA mutase
MCV	mean corpuscular volume
mg	miligram
MHz	megahertz
min	minutes
ml	mililitre
mm	milimetre
MMA	methylmalonic acid
MPV	mean platelet volume
NH_3	ammonia
NMR or H-NMR	nuclear magnetic resonance
p	p-value
PC	phosphatidylcholine
PCA	principle component analysis
PDW	platelet distribution width
PE	phosphatidylethanolamine
PLS-DA	partial least squares disciminant analysis
ppm	parts per million
PQN	probabilistic quotient normalisation
PS	phosphatidylserine
Q2	cross-validated sum of squares R2
RBC	red blood cell
RDW	red blood cell distribution width
RNA	ribonucleic acid
rpm	revolutions per minute
s	seconds
SAM	S-adenosylmethionine
SCFA	short chain fatty acids
SD	standard deviation
SHMT	serine hydroxymethyltransferase
THF	tetrahydrofolate
TMA	trimethylamine
TMAO	trimethylamine oxide
TSP	total serum protein
UDPG	uridine 5'-diphosphate glucose
µg	microgram
µl	microlitre
µM	micromolar
µmol	micromole
UMP	uridine monophosphate
VIP	variable importance in projection
WBC	white blood cell

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Supplementary Figure S3. A two dimensional PCA (left) and PLS-DA (right) scores plot of the metabolites identified in the serum of captive cheetahs at baseline (top panel), on the control (middle panel) and glycine supplemented (bottom panel) diets. The combined n=10 group is reflected in blue and the split n=6 group is reflected in red. *Each point represents an individual cheetah with a corresponding 95% confidence ellipse for each group.*

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ABSTRACT

Cheetahs are a vulnerable species and their conservation is of global importance. In captivity, cheetahs are prone to a wide range of gastrointestinal diseases that are believed to be linked to the diets they receive in captivity. In the wild, cheetahs eat whole carcass diets rich in collagen. This is not always feasible in captive settings resulting in most facilities feeding their cheetahs only raw muscle meat, which has lower collagen concentrations. Glycine is a simple amino acid that has many physiological functions in mammals, most notably its role on collagen biosynthesis, where it is the most abundant amino acid present. Anecdotal evidence showed that dietary glycine supplementation may improve gastritis. Thus, this study aimed to determine the effect of glycine supplementation on the metabolism of the cheetah. Ten healthy male and female captive cheetahs housed at Cango Wildlife Ranch in Oudtshoorn, Western Cape were fed either a meat only or glycine supplemented meat diet for four weeks, followed by a 4 week cross-over. Body measurements, urine and blood samples were collected at baseline, four weeks (diet 1) and eight weeks (diet 2). Haematology, serum biochemistry and untargeted ^{1}H nuclear magnetic resonance analysis was performed followed by the appropriate spectra, data and statistical analyses. The glycine diet resulted in a decreased serum albumin, alkaline phosphatase and total calcium concentration and an increased eosinophils and basophils count compared to the baseline or control diet. Body weight also decreased on the glycine diet which may be due to shifts in energy metabolism when glycine was supplemented. A total of 151 and 60 metabolites were identified in the urine and serum, respectively, in the 10 cheetahs. After data reduction, 10 and 7 metabolites were highlighted as important contributors towards the metabolome differences between the diet groups. The important metabolites identified in urine were dimethyl sulphone, proline, fructose, dimethylamine, trimethylamine, pyroglutamic acid, 1,3-diaminopropane, dihydrothymine, methylmalonic acid and pimelic acid. The metabolites identified in serum were glutamic acid, threonine, α-aminobutyric acid, glucose-6-phosphate, ethanolamine, methionine and propionic acid. These metabolites play various metabolic roles including energy production, immune function, protein and collagen biosynthesis or as products of gut microbiome fermentation. The collagen rich diet fed prior to the study, which was reflected in the baseline samples, had a more profound effect on the identified metabolites compared to the glycine supplemented diet. However, glycine supplementation did influence these pathways and had a direct effect on threonine and methionine related pathways. Specifically that of threonine sparing, pyrimidine biosynthesis and decreasing the production of bacterial fermentation products which may prove to be extremely beneficial in improving gastrointestinal health. This indicates the importance of this simple amino acid in whole body metabolism of cheetahs, especially when dietary collagen is limited. Glycine supplementation may be a simple method to improve the gastrointestinal and overall health of captive cheetahs and future studies should use targeted approaches to further understand the roles of the identified metabolites.

CHAPTER ONE

1. LITERATURE REVIEW

1.1. The cheetah *(Acinonyx jubatus)*

1.1.1. Background and the need for conservation

The cheetah (*Acinonyx jubatus*) is a unique felid highly specialised for the rapid pursuit of prey, reaching speeds of 29 m/s (104 km/h), and is the last remaining species of the genus *Acinonyx* (Durant et al., 2015; Hayward et al., 2006; Sharp, 1997). Adult cheetahs typically weigh between 25 to 65 kg, with males being larger for many morphometric measures compared to females (Marker and Dickman, 2003). Historically, cheetahs occupied a wide variety of habitats ranging from dry forests to grasslands across Africa and southwest Asia (Durant et al., 2015). Sadly, they now only occur in 9% of their historic range with an estimated 7100 adult and adolescent cheetahs distributed across 33 global population groups – of which half occur in a single population spanning six southern African countries (Durant et al., 2017). Cheetahs are currently listed as a vulnerable species on the International Union of Conservation of Nature (IUCN) Red List (Durant et al., 2015). According to Durant et al. (2017), the IUCN threat assessments are based on relatively well monitored populations within protected areas. For cheetahs, 67% of their population occurs on unprotected land which leads to increased threats, such as habitat loss, fragmentation, prey loss, human-wildlife conflict and illegal trade, with population densities rarely exceeding 0.02 individuals/km^2 (Durant et al., 2017). Additionally, the wide spread and elusive nature of the cheetah makes it extremely challenging to monitor them in unprotected areas and, therefore, declines within populations will go largely undetected. This along with the poor projection of growth rates when using a model of cheetahs located in protected areas, suggests that cheetahs should be categorised as endangered under the IUCN Red List (Durant et al., 2017).

Along with the threats experienced by cheetahs in protected and unprotected areas (such as land loss, prey loss and predation by larger predators) the limited genetic diversity of the cheetah also contributes to their vulnerability (Durant et al., 2017; O'Brien et al., 1985). In a 1985 study, the semen samples from 18 cheetahs were analysed and compared to domestic cats (O'Brien et al., 1985). It was found that cheetahs had lower spermatozoal concentrations and that 71% of all examined spermatozoa were morphologically abnormal compared to 29% in domestic cats. An earlier study investigated 200 isozyme and protein loci in 55 cheetahs and found that they contain up to 100 times less genetic variation than other mammalian species (O'Brien et al., 1983). These studies point to a severe population bottleneck in the cheetah's recent evolutionary history which has led to such high genetic uniformity (O'Brien et al., 1985). This genetic uniformity allows the expression of deleterious recessive alleles and limits adaptive ability to changes in their ecological

niche. Additionally, cheetahs have a notoriously high juvenile mortality rate in captivity which has made breeding difficult (O'Brien et al., 1985). Due to the vulnerability of the cheetah, it is necessary to focus on their management and conservation.

1.1.2 Brief anatomy

The remarkable anatomy and morphology of the cheetah enables it to reach high speeds rapidly. Adaptations include the long, slender legs, high respiratory, cardiovascular capacity, specialised muscle for high acceleration, pointed foot pads, semi- retractile claws, aerodynamic frame and a small skull with large sinuses for airflow (Marker and Dickman, 2003; O'Brien et al., 1985; Williams et al., 1997). The fibre type composition and metabolic enzyme activity of the skeletal muscle in cheetahs also highlights their speed capabilities with a predominance of the fast twitch type IIX fibres, a reliance on the anaerobic pathway and rapid hydrolysis of creatine phosphate for ATP regeneration (Williams et al., 1997).

In a comprehensive study by Hudson et al. (2011a), the hindlimb musculoskeletal anatomy of the cheetah was examined to provide insight into their locomotor capabilities and compared to racing greyhounds. The cheetah had longer hindlimb bones which would lead to increased stride length, a smaller volume of hip extensor muscles potentially indicating that the power for acceleration is not produced by the hip but by the vast back musculature. Lastly, they found that the cheetah had a large psoas muscle (links the lower back to the femur through the pelvis) which the authors believe enable rapid protraction of the hindlimb and resists certain forces generated around the hip during acceleration. In a similar study looking at the forelimb musculature, they found that that the total muscle mass of the forelimbs represented 15.1 ± 1.2% of the total body mass, while the hindlimbs represented 19.8 ± 2.2% (Hudson et al., 2011b). This may indicate that the forelimbs play a greater role in deceleration, while the hindlimbs accelerate the centre of mass. The cheetah also had longer forelimb bones than the greyhound which has similar implications to the longer hindlimbs. Lastly, the digital flexor muscles were larger which they hypothesise is an adaptation to aid in the protraction of the dew claw to capture prey. These studies highlight the unique anatomy of the cheetah for speed, however, the trade-off is that their smaller stature make them vulnerable to either being killed or for their prey to be stolen by more powerful predators such as lions and hyenas (Hunter et al., 2007; Marker and Dickman, 2003).

1.2. Cheetahs in captivity

1.2.1. History and management

Of the big cats, cheetahs are the easiest to tame and adapt quickly to captive settings. As a result, they have a long history with humans dating back to Samaria 3000 BC (Marker-Kraus, 1997; Woc Colburn et al., 2018). The earliest record of a captive cheetah is in 1829 at the Zoological Society

of London with the first confirmed captive birth being recorded many years later in 1956 at the Philadelphia Zoo (Marker-Kraus, 1997). Breeding in captivity is notoriously challenging due to a combination of their genetic uniformity and high infant mortality rates, with only limited facilities having success with limited males and females (Marker-Kraus, 1997; O'Brien et al., 1985). However, when facilities incorporate correct breeding management programmes this may drastically improve the success and sustainability of breeding in captivity as was demonstrated by the de Wildt Cheetah and Wildlife Centre in South Africa in which 785 cubs were born between 1975 to 2005 with a survival rate of 71% (before 12 months) and 66% (after 12 months) (Bertschinger et al., 2008). According to the latest International Cheetah Studbook (2020), the captive population consists of 1851 individuals in 272 known facilities across 46 countries. The United States of America and South Africa are the largest contributors to the captive population with 486 and 381 individuals, respectively (Marker and Johnston, 2020). These reported numbers have likely increased since the publication in 2020.

The correct management of captive cheetahs is vital in maintaining a healthy population. There are various aspects that need to be considered, including the housing requirements, stress management, restraint and handling, health examinations and hand rearing of cubs (Woc Colburn et al., 2018). Additionally, enrichment programmes that aim to enhance the quality of life by providing stimuli that promote natural and active behaviours are common in these facilities, and include changing the structure or content of enclosures or changing husbandry practices such as feeding times (Quirke and O' Riordan, 2011).

1.2.2. Diseases of captive cheetahs

The understanding of disease is important in maintaining and managing the captive cheetah population. In 1993, a comprehensive pathology survey was conducted in the North American captive cheetah population to determine the prevalence of disease (Munson, 1993). Of the 31 deceased adult cheetahs included in the survey, veno-occlusive disease (fibrosis or occlusion of hepatic sinusoids or veins) directly accounted for the death of 9 individuals and was present in 82% of the cheetahs, while the renal disease, glomerulosclerosis, accounted for the death of 8 individuals and, similarly, had a prevalence of 84%. Chronic lymphoplasmacytic gastritis (inflammation of the gastric muscosa) was present in 91% of the cheetahs and believed to be caused by spiral bacteria (*Gastrospirillum sp.* and *Helicobacter pylori*). These bacteria were also present in a corresponding study investigating the role of bacteria in chronic gastritis (Eaton et al., 1993). The authors concluded that the presence of one or both of these bacteria provided evidence that the cause of gastritis is bacterial. However, this was later refuted as a high prevalence of *Helicobacter sp.* was also found in free-ranging cheetah populations who showed no signs of gastritis (Munson et al., 2005).

In a South African captive cheetah population, gastritis had a prevalence of 100% followed by adrenal cortical hyperplasia (83%), glomerulosclerosis (80%), splenic lymphocytic depletion (73%) and veno-occlusive disease (36%) (Munson et al., 1999). The presence of the same unusual diseases, namely chronic lymphoplasmacytic gastritis, glomerulosclerosis and veno-occlusive disease, in two separate populations indicates that the cause may be due to genetic or environmental factors. Munson et al. (2005) then investigated a population of free-ranging Namibian cheetahs for the same unusual diseases. Interestingly, the free-ranging population was largely disease free with a much lower prevalence of glomerulosclerosis (13%), gastritis (11%) and veno-occlusive disease (8%) compared to their captive counterparts. The absence of these significant health problems in the free-ranging populations indicates that genetics alone cannot be the main factor determining the health and fitness of cheetahs, as the Namibian free-ranging population would form part of the historical founding population for North American and South African captive populations (Marker-Kraus, 1997). The higher prevalence of adrenal cortical hyperplasia in the captive population may indicate a physiological stress response to captivity (Munson et al., 2005, 1999). Munson et al. (2005) postulated that the lack of genetic diversity of the cheetah has limited their capacity to adapt to the captive environment resulting in a chronic stress response that exacerbates disease development by changing normal physiological homeostasis.

The high morbidity and mortality due to renal disease, such as glomerulosclerosis and medullary fibrosis, has been positively associated with age in captivity affecting cheetahs over the age of seven years (Mitchell et al., 2018). Medullary fibrosis was notably more severe in the outer third of the renal medulla where metabolic activity is highest and relative hypoxia is greatest. This led the authors to conclude that chronic stress levels may lead to constant activation of the sympathetic nervous and renin angiotensin aldosterone systems (which may also be linked to a NaCl deficiency in the diet) which diverts blood to the brain, muscle and heart at the expense of the kidney and liver. This may lead to repetitive subclinical hypoxic injury to the renal medullary tubules, contributing to the development of chronic renal disease in cheetahs (Mitchell et al., 2018). Many of the other unusual disease as well as the general disease burden (count of the number of common lesions) has been positively associated with age (Gillis-Germitsch et al., 2017). Interestingly, disease burden and gastritis were only moderately associated with adrenal morphology indicating that it is partly stress related, but that the relationship is likely multifactorial (Gillis-Germitsch et al., 2017).

Various aspects of captivity, including stress, diet, age and lack of physical activity may be contributing to the array of unusual diseases experienced by captive cheetahs compared to their free-ranging counterparts (Gillis-Germitsch et al., 2017; Mitchell et al., 2018; Munson et al., 2005; Woc Colburn et al., 2018). The metabolic and physiological mechanistic response of the cheetah to these various aspects of captivity and disease development is largely unknown.

1.2.3. Stress in captivity

The high prevalence of unusual disease in captive, but not free ranging cheetahs, as well as the high prevalence of adrenal cortical hyperplasia has led researchers to believe that the stress experienced in captivity is a major contributor (Munson et al., 2005, 1999). Chronic or repeated stress exposure leads to persistent adrenocorticotropic hormone (ACTH) release from the pituitary gland which may lead to hypertrophy and hyperplasia of the zona fasciculata of the adrenal cortex, and therefore, an enlarged adrenal cortex has been used as a morphological indicator of chronic stress (Terio et al., 2004). Terio et al. (2004) compared the adrenal gland morphology with the faecal cortisol concentration of captive and free-ranging cheetahs and found that captive cheetahs had a higher cortisol concentration and larger adrenal cortices than free ranging cheetahs. The faecal cortisol concentration was then investigated over 30 days in captive cheetahs moved between “on-exhibit” and “off-exhibit” sites, in which the former allows for public viewing (Wells et al., 2004). Individuals transferred from “off-exhibit” to “on-exhibit” showed a trend towards having higher and more prolonged stress responses compared to those housed “off-exhibit”. The same observation was recorded by Terio et al. (2004). However, there was notable individual and daily fluctuation in cortisol concentrations in both of these studies which may indicate that individual animals respond differently to environmental changes (Terio et al., 2004; Wells et al., 2004). This also highlights that blood and faecal glucocorticoid concentrations may be influenced by other factors like age, sex and diet, and does not necessarily show the full extent of physiological stress experienced by these animals (Kirberger and Tordiffe, 2016).

The use of trans-abdominal adrenal gland ultrasonography and blood markers of oxidative damage, has been suggested as alternative methods to determine the stress response (Costantini et al., 2017; Kirberger and Tordiffe, 2016). Kirberger and Tordiffe (2016) found that the adrenal glands were easily identified and measured, with the right adrenal gland slightly more difficult to locate. Therefore, the left adrenal gland should be used in future studies, as measurements of the various adrenal dimensions may accurately reflect long term stressors. An advantage of this method is that it could also provide the opportunity to visualise and detect pathology of other abdominal organs (such as the kidney and liver) during a routine health check. In the past 10 years, there has been an increasing interest in molecular oxidative damage and antioxidant mechanisms of free-ranging animals and its relationship with environmental stressors and important life-history functions (Costantini et al., 2019, 2017). Blood markers of oxidative damage and antioxidant capacity increased when cheetahs were physically restrained in box traps, indicating that these parameters are sensitive to acute stress responses (Costantini et al., 2017). Additionally, as captive cheetahs showed higher levels of oxidative damage markers but no corresponding clinical signs of disease, the authors suggested that the higher level of reactive oxygen metabolites and protein carbonyls may be linked to the high intake of polyunsaturated fatty acids in captivity, which are quickly oxidised to produce free radicals (Costantini et al., 2017; Tordiffe et al., 2016). A later study by the

same authors, showed that free ranging female cheetahs had higher levels of oxidative stress than male cheetahs, and male and female leopards occupying the same areas (Costantini et al., 2019). They believed this was due to the cumulative lifetime costs of reproduction for female cheetahs who produce six cubs on average for each reproductive event versus the two cubs produced on average for female leopards. These studies emphasizes the need to use alternative metabolic and physiological tools to uncover unnoticed costs that might be negatively affecting the fitness of threatened species.

1.2.4. Physical activity in captivity

In their natural distribution ranges, cheetahs cover wide areas, with home ranges in excess of 3000 km^2, while in captivity enclosure sizes can vary depending on the facility with a recommended minimum surface area of 750 m^2 housing two cheetahs (Durant et al., 2017; Woc Colburn et al., 2018). Therefore, the potential physical activity of free-ranging cheetahs would be markedly higher than their captive counterparts. Lack of physical activity has been stated as a potential contributor to the prevalence of various diseases within the population (Munson et al., 2005). In captive settings, the practise of enrichment would provide cheetahs with the best opportunity to do their natural active behaviours such as running, chasing and exploring (Quirke and O' Riordan, 2011). A common practice at different facilities would be lure-coursing or the "cheetah run" which uses a pulley system to move "prey" (usually a white rag) around a track to promote hunting behaviour (Quirke et al., 2013). Aside from the behavioural and management benefits, the physiological benefit of physical activity in captivity is unknown and should be an area of future research.

1.2.5. Diets in captivity

Cheetahs are obligate carnivores and, in the wild, consume bones, tendons and cartilage on a regular basis (Depauw et al., 2013). Cheetahs generally pursue small to medium sized prey with a similar body mass to themselves, ranging between of 23 to 56 kg, in order to optimise their success rate, limit injury and consume their meal before kleptoparasites arrive (Hayward et al., 2006). This makes them very efficient hunters with over 50% of pursuits ending in kills (Bissett and Bernard, 2007; Hayward et al., 2006). Common prey species include impala, blesbok, Grant and Thomson gazelle, springbok and common duiker (Hayward et al., 2006). Male coalitions will generally take on larger prey such as kudus, while single females or those with cubs will pursue smaller antelope such as common duiker or springbok (Bissett and Bernard, 2007).

In a global survey conducted by Whitehouse-Tedd et al. (2015) encompassing 86 facilities representing 12% of the captive cheetah population, it was found that a raw meat diet (37%) was the most popular diet. This was followed by a commercially prepared diet (20%) and carcass diet (8%), with 35% of cheetahs receiving a mixture of the three diets. Interestingly, commercially prepared diets were only provided in North American facilities, while raw meat was available

globally. This was also observed in an earlier study where 13 North American facilities fed on average 1.3 ± 0.4 kg per day commercially prepared frozen canine or feline diets (Dierenfeld, 1993). Additionally, whole or partial carcasses of rabbits, venison and chickens were fed once or twice a week. Raw meat diets mostly consisted of muscle meat (beef, horse, chicken etc.) with a carcass component (such as hide, skin, bone, fur, feather, feet or wing) included at least once a month (Whitehouse-Tedd et al., 2015). Vitamin and mineral supplementation were provided to 53% of captive cheetahs. The raw meat diet had a greater association with firm and dry faeces (considered "normal") while the carcass only diet was linked to extremely dry faeces – this may provide an indicator of gastrointestinal health (Whitehouse-Tedd et al., 2015). The different diets in captivity may also play a role in psychological wellbeing and enrichment, with carcass diets keeping cheetahs active and interested for longer periods of time compared to commercial diets (Bond and Lindburg, 1990).

With high variability in diet seen across free-ranging and captive cheetahs, facilities and continents, and the potential link to gastrointestinal and other disease, it becomes increasingly important to further understand the role of each dietary component on cheetah health.

1.3. Nutrition and metabolism

There have been comprehensive studies done in the domestic cat, and therefore, they are often used as a model of nutrient requirements in exotic felids. Indeed, when domestic cats and large exotic felids (including the cheetah, jaguar, Malayan and Amur tigers) where fed the same beef or horse based diet, they had very similar digestibility responses indicating that the domestic cat is an appropriate model (Vester et al., 2010). An overview of the amino acid and fatty acid energy production pathways are shown in Figure 1.

1.3.1. Protein

As a strict carnivore, dietary protein makes up a high percentage of the captive cheetahs diet with a crude protein range of 55 to 86% depending on the meat source and additional supplementation (Bechert et al., 2002; Depauw et al., 2012b; Dierenfeld, 1993). An animal's protein requirement is a reflection of the minimal nitrogen and amino acid requirement, most of which are used for energy production (MacDonald et al., 1984). Regardless of whether cats are fed a high or low protein diet, there is very little adaptation of aminotransferase or urea cycle enzymes, therefore they continue to use protein for energy production or in other metabolic pathways even with limited availability (Zoran, 2002). Virtually the same amino acids are essential for all species – arginine, histidine, isoleucine, lysine, leucine, methionine, phenylalanine, threonine, tryptophan and valine (MacDonald et al., 1984). However, the cat requires specific amino acids (taurine, arginine, methionine and cysteine) in their diet which are not produced endogenously (Zoran, 2002). A single meal devoid of arginine can lead to hyperammonemia within a few hours. The increased sensitivity

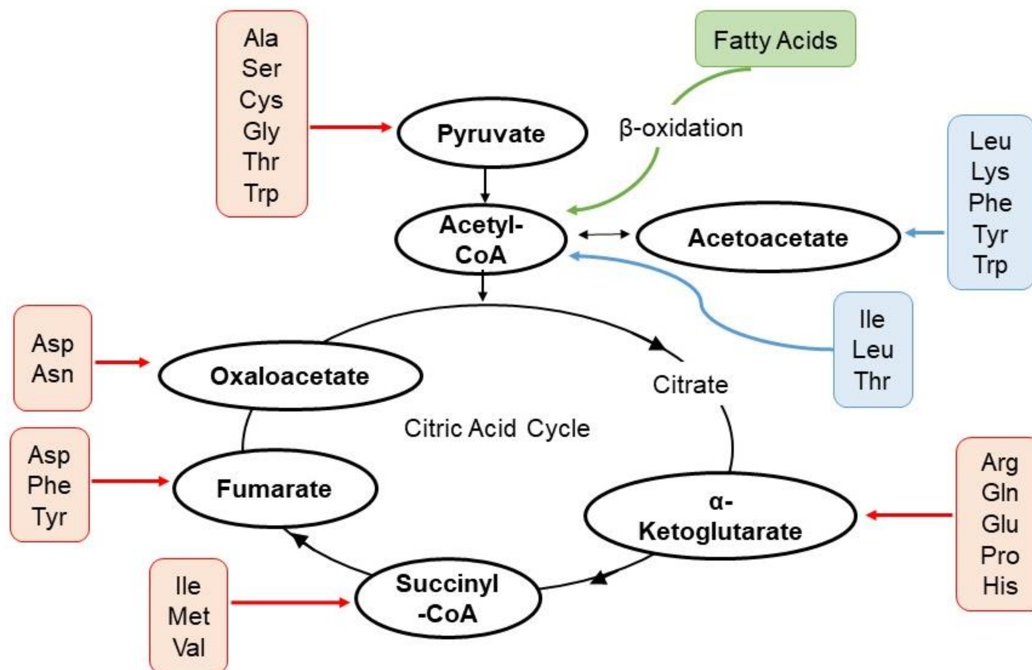

Figure 1. An overview of the role of fatty acids (green), gluconeogenic amino acids (red) and ketogenic amino acids (blue) in energy production. Adapted from Garrett and Grisham (2012).

to arginine deficiency is likely due to the lower ornithine biosynthesis (from glutamate) and less citrulline provided to the kidney for conversion to arginine (MacDonald et al., 1984). A dietary supply of taurine is also required, as inadequate amounts are produced from methionine and cysteine precursors, and a deficiency will lead to central retinal degeneration (MacDonald et al., 1984; Zoran, 2002). In addition to its conversion to taurine, cats have a higher requirement of cysteine and methionine because these gluconeogenic amino acids are catabolised to pyruvate and succinyl-CoA, respectively, in the citric acid cycle (Zoran, 2002). Tryptophan has limited movement through the quinolinic acid pathway and therefore does not synthesise enough niacin (vitamin B_3 or nicotinic acid) in the cat (MacDonald et al., 1984). Tordiffe et al. (2019) used an untargeted exploratory gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) approach to determine the serum and urine amino acid profiles of captive cheetahs. In serum, 38 amino acids were identified with glutamine (1624 µmol/L), alanine (772 µmol/L), arginine (670 µmol/L) and glycine (479 µmol/L) having the highest concentrations. Similarly, 38 amino acids were identified in urine with arginine (740 µmol/L), glutamine (437 µmol/L), alanine (397 µmol/L) and serine (331 µmol/L) having the highest concentrations.

1.3.2. Carbohydrates and fats

Carbohydrate ingestion in cheetahs is extremely low and would make up a negligible portion of any commercial, carcass or supplemented meat based diet (Bechert et al., 2002; Dierenfeld, 1993). However, when fed carcass diets, connective tissue may contribute to the dietary carbohydrate portion as collagen covalently binds glucose and galactose (Depauw et al., 2012a). Excess

carbohydrates that are not enzymatically digested in the small intestine will provide substrates for increased microbial fermentation in the large intestine (Depauw et al., 2012a; Zoran, 2002). Domestic cats lack salivary amylase to initially breakdown the ingested carbohydrates, have low hepatic glucokinase activity to phosphorylate glucose to glucose-6-phosphate and have low glycogen synthase activity to synthesise glycogen from glucose (Zoran, 2002).

After protein, fat makes up a substantial proportion of the cheetah's diet with a range of 9% to 26% crude fat depending whether the diet is meat or carcass based (Bechert et al., 2002; Depauw et al., 2012b). The three major functions of dietary fat is (1) as a concentrated energy source, (2) to provide essential fatty acids and (3) as a carrier for fat soluble vitamins (MacDonald et al., 1984). Fatty acids are either saturated or unsaturated and consists of a carbon chain between 2 to 36 carbons long which can be synthesised in the cytoplasm by NADPH and Acetyl-CoA-carboxylase (Tvrzicka et al., 2011). Their degradation in the mitochondria by β-oxidation leads to the production of ATP for energy. Saturated fats do not contain any double bonds and vary from short chain (including acetic acid, butyric and propionic acid) to long chain (including palmitic and stearic acids). Unsaturated fatty acids, which contain double or triple bonds, can be broken into monounsaturated and polyunsaturated fatty acids (Tvrzicka et al., 2011). The latter contains the essential fatty acids α-linolenic acid and linoleic acid, which are then further elongated and desaturated to form eicosapentaenoic acid, docosahexaenoic acid and arachidonic acid. Cats, and likely cheetahs, have limited Δ-6-desaturase activity and other hepatic desaturase activity and are unable to produce arachidonic acid which is a dietary essential fatty acid in cats (MacDonald et al., 1984). Tordiffe et al. (2016), investigated the serum fatty acid profile of free-ranging and captive cheetahs using GC-MS and found that there was a decreased concentration of monounsaturated, polyunsaturated and total fatty acids in free-ranging cheetahs compared to their captive counterparts. This noticeable profile difference is likely due to differences in dietary fatty acid composition and fatty acid metabolism.

1.3.3. Vitamins and minerals

The fat soluble vitamins A and D are found in animal tissue (such as the liver) and their content should be met in the natural carnivorous or meat based diets of the cheetah (Zoran, 2002). The water soluble vitamin, niacin or nicotinic acid, cannot be synthesised in the cat as they cannot convert tryptophan via quinolinic acid to niacin and it is therefore required in the diet (MacDonald et al., 1984). They also require thiamine, riboflavin, pantothenic acid, pyridoxine, biotin, folacin and vitamin B_{12} from the animal tissue in their diet. The mineral requirement (e.g. magnesium, potassium, sodium, zinc etc) would be fairly similar across species and should be obtained from the diet (MacDonald et al., 1984). Lack of bones in the captive diet may lead to calcium and phosphorous deficiencies which would require additional supplementation.

1.4. Dietary studies in cheetahs

An early study into the dietary composition of commercial feline diets fed to captive cheetahs in North America, found that these diets had two major isoflavones present which were absent in chicken and horse meat diets (Setchell et al., 1987). Daidzein and genistein, which have structural similarities to steroidal oestrogens and synthetic steroids, were found in the soybean component of the commercial diet. The authors hypothesised that cheetahs may be sensitive to these dietary oestrogens due to the poor hepatic conjugation of xenobiotics and phenolic compounds which is crucial for inactivation and excretion, and this may account for the poor reproductive success of captive cheetahs (Setchell et al., 1987).

Since 1987, there have been a number of studies investigating the nutritional composition of the various diets that captive cheetahs are fed in order to elucidate the potential cause of the high disease rate in captivity (Bechert et al., 2002; Depauw et al., 2013, 2012b, 2012a; Gosselin et al., 1989; Lane et al., 2012; Vester et al., 2008). Gosselin et al. (1989) compared the nutritional composition of eight different commercially prepared diets, ranging from horse meat, chicken, beef and different combinations of vitamins and minerals, using high performance liquid chromatography (HPLC) and GC-MS. The authors noted that the vitamin A levels fluctuated significantly between diets where they were either excessive or deficient based on the recommended dosage and may be a contributor to the veno-occlusive disease seen in captive cheetahs. Similarly, Bechert et al. (2002) compared a commercial diet with five supplemented vitamin and mineral muscle meat diets (cattle, horse, donkey, turkey and deer). The commercial diet had one to five times the level of recommended nutrients, notably vitamin A, while overall the supplemented muscle diets were deficient in vitamin E and copper. The protein to fat ratio was 6:1 for the supplemented meat diets (68 - 84% crude protein), while the ratio for a whole carcass meal is reportedly 3:1 – this high protein content may account for the elevated blood urea nitrogen (BUN) and creatinine concentrations (Bechert et al., 2002). The supplemented meat diets were also lower in fat, and authors suggested that they may require additional essential fatty acid supplementation. Plasma zinc was the only significantly different nutrient between cheetahs, with the supplemented meat diet yielding higher concentrations. These results were also echoed in a later study where cheetahs fed a supplemented beef diet had a higher zinc and lower vitamin A concentration than individuals fed a whole rabbit diet (Depauw et al., 2012b). Although the whole rabbit diet had a higher fat content, both diets led to a deficiency in essential fatty acids, such as linoleic acid and arachidonic acid. Another concern was the high serum levels of vitamin A from the whole rabbit diet, likely due to consumption of the liver, after just four weeks of the diet. The authors concluded that the exclusive use of a single long term food source is not recommended for captive cheetahs.

The diet of captive cheetahs generally consists of high amounts of protein. Excess protein in the diet may result in increased amounts of undigested protein which can lead to bacterial fermentation

in the intestine producing more detrimental by-products such as ammonia, indolic and phenolic compounds (Depauw et al., 2013; Vester et al., 2008). Vester et al. (2008) investigated the nutrient digestibility, faecal characteristics and faecal fermentation end product concentrations of five exotic captive felids (bobcat, jaguar, cheetah, Siberian tiger and Indochinese tiger) fed the same commercially prepared beef diet. The cheetah had a lower fat digestibility and higher concentrations of faecal ammonia and indole compared to the other species. This highlights that different diets should be considered for different exotic felids in captivity, but also that captive cheetahs produce greater concentrations of putrefactive or detrimental by-products compared to their felid counterparts. Similarly, in a cross-over study, cheetahs fed a supplemented beef diet had a higher faecal concentration of short chain fatty acids (SCFA; propionic acid and butyric acid), branched chain fatty acid (BCFA; isobutyrate and isovalerate) and putrefactive compounds (indole, phenol and p-cresol) compared to a whole rabbit diet (Depauw et al., 2013). This was somewhat surprising as the connective tissue found in the rabbit diet is considered to be poorly digestible thus leading to a higher fermentation rate and more putrefactive compounds. The authors concluded that the indigestible parts of the rabbit (bone, cartilage and hair) may act as a bulking agent in the intestine creating a barrier between the bacteria and the substrate which they termed “animal fibre”. When investigating the *in vitro* kinetic fermentation rates of different animal substrates, glucosamine and glucosamine-chondroitin (saccharides that make up cartilage) were highly fermentable (Depauw et al., 2012a). This was followed by the moderately fermentable chicken cartilage and collagen, while rabbit hair, skin and bone were poorly fermentable. Therefore, the presence or absence of certain animal tissues may cause changes in digestion and intestinal fermentation which could play a role in the gastrointestinal health of the cheetah (Depauw et al., 2013, 2012a). Indeed, cheetahs fed a commercial diet over a two year period took longer to develop gastritis or renal disease than cheetahs fed a supplemented horse meat diet (Lane et al., 2012). Although the diet and health status of the cheetahs were variable and no serum or faecal analysis was performed, this study still highlights the need to further understand the role of diet in disease progression and overall health of the cheetah.

1.5. The role of glycine – a simple amino acid

Glycine is the simplest amino acid and is traditionally classified as “nutritionally non-essential” as it is synthesised endogenously in the body (Wang et al., 2013). It was first isolated in 1820 by French Chemist Braconnot and named after its sweet taste. Glycine plays a key role in the metabolism, cytoprotection, immune response, growth and development of many mammals and humans, and represents 11.5% of the total amino acid content in humans (Razak et al., 2017). An overview of glycine biosynthesis, degradation and its various physiological roles are provided in Figure 2.

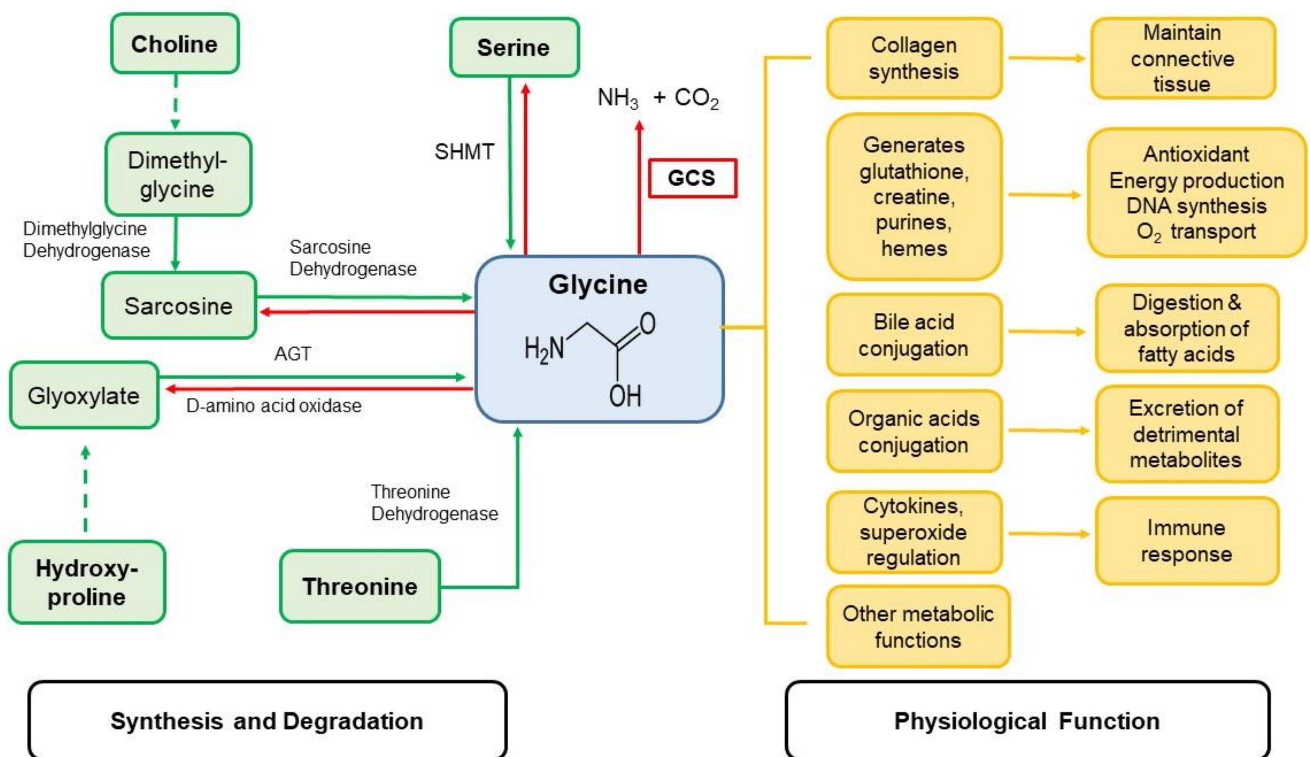

Figure 2. A schematic diagram of glycine biosynthesis, degradation and various physiological roles in mammals. AGT, Alanine:glyoxylate aminotransferase; SHMT, Serine hydroxymethyltransferase; GCS, glycine cleavage system. Adapted from Meléndez-Hevia et al. (2009); Wang et al. (2013).

1.5.1. Glycine biosynthesis

Glycine is synthesised endogenously via four pathways. Firstly, glycine can be synthesised from serine obtained from the diet or produced endogenously via glucose and glutamate (Wang et al., 2013). Serine hydroxymethyltransferase (SHMT), which catalyses the reaction, is found in the cytosol and mitochondria of cells. However, the mitochondrial SHMT is ubiquitous in the body and responsible for most of the conversion of serine to tetrahydrofolate which yields glycine and N5-N10-methylene tetrahydrofolate. Secondly, glycine can be synthesised from threonine via threonine dehydrogenase (Wang et al., 2013), but this is a minor source of glycine and is dependent on the dietary intake of threonine.

During the oxidative degradation of choline, an essential nutrient, to betaine, the three methyl groups of choline are released and involved in other reactions leading to the production of glycine (Wang et al., 2013). Firstly, betaine is used as the methyl donor converting homocysteine into methionine which produces dimethylglycine. Dimethylglycine is then converted to sarcosine (N-methylglycine) by dimethylglycine dehydrogenase, before being converted to glycine by sarcosine dehydrogenase. Sarcosine and glycine are interconvertible and this is regulated by sarcosine dehydrogenase. Although 40 to 70% of ingested choline may be converted to glycine (which is the case in adult rats), generally, the choline content of diets are quite low in animals and are particularly important when the dietary methionine is minimal (MacDonald et al., 1984; Wang et al., 2013).

The final pathway for glycine biosynthesis is via hydroxyproline which is formed from the post translation hydroxylation of proline in collagen biosynthesis (Li and Wu, 2018). In the mitochondria, hydroxyproline is broken down to glyoxylate which undergoes transamination via Alanine:glyoxylate aminotransferase (AGT) to form glycine and pyruvate (Wang et al., 2013). It is evident that glycine is an important amino acid as there are multiple pathways to synthesise it. However, evidence has shown that the amount of glycine synthesized *in vivo* is not enough to meet metabolic demands in humans, pigs and rodents (Wang et al., 2014, 2013).

1.5.2. Glycine degradation

The catabolism of glycine mainly takes place in the small intestine and is a preferred substrate for many intestinal bacteria such as *Escherichia coli* (Dai et al., 2011). The glycine cleavage system (GCS) is a complex enzyme system consisting of three proteins and one carrier protein, found in the mitochondria of most species. This complex is responsible for the deamination and decarboxylation of glycine producing CO_2, NH_3 and tetrahydrofolate. This reaction couples with SHMT, as tetrahydrofolate is an essential cofactor, to convert glycine into serine which is a reversible reaction (Wang et al., 2013). Therefore, glycine can either be converted to serine or degraded to CO_2 and NH_3 via GCS. GCS favours the oxidation of glycine and not its synthesis, and is crucial when metabolic acidosis, high protein diets, and glucagon lead to an increased glycine degradation (Razak et al., 2017). Lastly, glycine can be converted to glyoxylate through D-amino acid oxidase, although this degradation process would be highly insignificant (Wang et al., 2013).

1.5.3. Physiological benefits of glycine

Glycine has many physiological functions. The most notable is its role, along with proline and hydroxyproline, in the biosynthesis of collagen (Li and Wu, 2018). Collagen is the largest and most abundant protein in the body, accounting for one third of the total proteins in animals, with glycine contributing to every third position of the collagen triple helix. Collagen provides the strength, rigidity and flexibility of connective tissue found in bones, skin, cartilage and blood vessels, and is therefore crucial in the growth, development and health of all animals (Li and Wu, 2018).

Glycine also provides flexibility at the active sites of many enzymes, generates glutathione (major antioxidant), creatine (involved in muscle and nerve metabolism), purines (DNA synthesis), haem (oxygen transport) and serine (Wang et al., 2013). Creatine or phosphocreatine is an important rapid energy provider in tissue such as skeletal muscle, cardiac muscle, brain, the retina and spermatozoa (Wyss and Kaddurah-Daouk, 2000). The high speed lifestyle of cheetahs undoubtedly adds to the importance of creatine as a fuel source (Williams et al., 1997). The transfer of the amidino group of arginine to glycine yielding L-ornithine and guanidinoacetic acid is the first step in the biosynthesis of creatine and is considered the rate-limiting step of creatine biosynthesis. Along with the rapid energy requirement of the skeletal muscle of cheetahs, creatine and phosphocreatine

are the main energy providers for spermatozoa. Since creatine biosynthesis is dependent on glycine, the latter may have an impact on semen motility and, therefore, reproductive ability.

Glycine also plays a role in the digestion and absorption of lipids and lipid-soluble vitamins through the conjugation of bile acids. Also, glycine regulates the production of cytokines and acts as a neurotransmitter in the central nervous system (Wang et al., 2013). Lastly, several xenobiotic organic acids (such as benzoic acid and phenylacetic acid which are associated with neurological symptoms) are metabolised by conjugation to glycine in the mitochondria which allows them to be excreted from the body (Badenhorst et al., 2014).

1.5.4. Dietary glycine supplementation

As noted above glycine has many physiological and metabolic benefits, and chronic glycine shortage could lead to suboptimal growth, impaired immune response and other adverse metabolic effects (Wang et al. 2013). Meléndez-Hevia et al. (2009) used available literature to calculate various rates of glycine biosynthesis and consumption in humans, pigs, rats and rabbits and extrapolated it to an average 30 to 50 year old 70 kg adult human. They showed that the endogenous biosynthesis of glycine accounts for ± 3 g/day, and dietary glycine accounts for 1.5 to 3 g/day. This leaves a 10 g/day deficit for all the metabolic uses of glycine, including collagen biosynthesis. Therefore, glycine should be considered a "conditionally essential" amino acid with the appropriate dose of supplemented glycine generally considered safe and beneficial for humans and animals (Wang et al., 2013). Animal studies have used up to 3% glycine supplementation while clinical studies in humans have ranged between 3 g per day to 0.8 g/kg body weight per day with no adverse side effects which is likely due to the efficient absorption of glycine (McCarty et al., 2018; Petzke et al., 1986). Additionally, glycine is inexpensive, soluble and highly palatable due to its sweet taste, so it can easily be added to beverages or food (McCarty et al., 2018).

The effects of dietary glycine supplementation has been studied *in vivo* in rats, pigs, chickens, fish, rabbits and humans (Imenshahidi and Hossenzadeh, 2022; Li and Wu, 2018; McCarty et al., 2018; Wang et al., 2014). Briefly, glycine supplementation has been shown to increase plasma glycine and serine concentrations, decrease plasma ammonia, urea and glutamine concentrations, and increase oxidative defence in piglets (Wang et al., 2014). A high glycine diet in rats led to higher SHMT activity and CO_2 production from glycine catabolism, which indicates that glycine catabolism is dependent on and adapted to the glycine diet content (Petzke et al., 1986). In elderly humans, cysteine and glycine supplementation (which are the pre-cursors for glutathione synthesis) led to a 95% increase in glutathione concentration and a decrease in oxidative stress markers, which indicates that supplementation is a practical way to reduce oxidative stress caused by ageing (Sekhar et al., 2011). Plasma glycine concentrations are lower in individuals with metabolic syndrome (hypertension, diabetes etc.) while lifestyle and exercise interventions lead to an increase

in circulating glycine concentrations, indicating that glycine supplementation may improve various components of metabolic syndrome (Imenshahidi and Hossenzadeh, 2022). Supplemental glycine also effectively protects skeletal muscle in a variety of wasting models potentially due to receptor mediated response and modulation of intracellular metabolism (Koopman et al., 2017). There are a wide array of health benefits linked to glycine supplementation – controlling metabolic syndrome and diabetes, inhibiting atherosclerosis, reducing oxidative damage, improving sleep quality, collagen biosynthesis and promoting a healthy and optimum metabolism (Imenshahidi and Hossenzadeh, 2022; McCarty et al., 2018; Meléndez-Hevia et al., 2009).

While glycine supplementation has been investigated in a variety of smaller animals and humans, the effects have not been investigated in felid species such as the cheetah. Tordiffe et al. (2017) showed that a third of the 30 most abundant organic acids excreted in the urine of cheetahs is associated with phenylalanine, tyrosine and glycine conjugates. Additionally, cheetahs are prone to a variety of unusual gastrointestinal diseases in captivity which may be associated with their high protein diet. Excess protein in the diet or incomplete protein digestion can lead to bacterial fermentation in the intestine producing detrimental by-products such as ammonia, indolic and phenolic compounds (Depauw et al., 2013; Vester et al., 2008). Glycine plays a vital role in the conjugation of these compounds to allow urinary excretion (Badenhorst et al., 2014). This, together with their apparent need for large quantities of creatine, suggests that cheetahs have a substantial metabolic need for glycine which may not be met through endogenous biosynthesis or conventional captive diets as has been found in other species (Meléndez-Hevia et al., 2009). However, the metabolic implications of glycine supplementation in captive cheetahs are still unknown and warrant further investigation.

1.6. The metabolomics approach

Metabolomics is a rapidly evolving field of research that uses analytical chemistry techniques to comprehensively characterize the metabolome (Wishart, 2019). The metabolome is generally defined as the complete collection of metabolites found in a specific organelle, cell, organ, biofluid or organism. Metabolites are endogenous compounds such as lipids or amino acids that are produced by endogenous catabolism or anabolism. Although the metabolome is highly conserved across populations and species, an individual's metabolome is sensitive to internal and/or external variables – such as age, gender, diet, geographical location, time of day, environment and genetics (Wishart, 2019). Therefore, this metabolic phenotype is the product of an individual's genotype *and* environmental factors under certain conditions, and provides valuable information about what is happening within an individual as opposed to what might happen. This "top down" approach has many potential applications such as in personalised medicine, public healthcare, drug testing, nutrition analysis and veterinary science (Kim et al., 2016).

1.6.1. Overview of the metabolomics workflow

There are four key experimental designs to consider with metabolomics studies (Wishart, 2019). Firstly, the targeted approach which allows the identification of a small number of metabolites within a focused area of the metabolome and is generally for hypothesis testing. The untargeted approach is considered hypothesis generating as it allows the identification of as many metabolites as possible within the complex sample mixture (such as urine, serum, plasma etc.). Fluxomics, which measures the rate of metabolic reactions, and metabolite imaging, which allows for the *in vivo* or *in vitro* visualisation of metabolites, are the third and fourth approaches, respectively. These study designs can incorporate various methods to measure the metabolome, such as nuclear magnetic resonance (NMR), MS, LC-MS, GC-MS (Lenz and Wilson, 2007). Each method has its own advantages and disadvantages and the selected method would depend on the tools, time and funding available (Beckonert et al., 2007; Lenz and Wilson, 2007; Wishart, 2019). NMR, which was first utilised in the 1980s, provides structural information of small organic molecules based on atom-centred nuclear interactions and, although it is less sensitive than GC-MS and other MS methods, it is considered a robust, reliable and reproducible technique (Lenz and Wilson, 2007). NMR is inherently quantitative as the signal intensity is directly proportional to the number of nuclei present in the sample, this can either provide a relative or absolute quantification (Crook and Powers, 2020). Relative quantification is the measurement of molar concentrations relative to control samples or groups within a study which is determined by multivariate and univariate analyses of NMR spectral data. On the other hand, absolute quantification is the direct measurement of individual compound concentrations using internal standards or metabolite deconvolution (i.e. separation of molecules achieved computationally or experimentally) (Crook and Powers, 2020).

Once raw spectral data has been obtained via the selected method, the next challenge would be processing of this data by various software packages to allow for the identification and quantification of the metabolites within the complex sample mixture (Hendriks et al., 2011). Due to the rapidly growing field, the available software packages or tools are constantly improved and updated – roughly 85 new metabolomics software resources, tools, packages and databases were introduced in 2020 (Misra, 2021). Many of these packages are incorporated in developing software, such as R or Python, or are web based, and provide varying degrees of pre-processing, processing, annotation, metabolite identification and metabolite quantification from the raw data obtained from different platforms (such as GC-MS, NMR). Both univariate and multivariate statistical analyses can then be performed on the quantitative metabolite data (Saccenti et al., 2014). Univariate analysis (e.g. t-test, ANOVA) allows the comparison of one variable and focuses on the independent changes in metabolites, whereas multivariate analysis (e.g. correlations, linear regression) allows the comparison of two or more variables simultaneously, focusing on the relationship between metabolites and their complimentary behaviour in relation to biological processes (Saccenti et al., 2014). Both analytical methods should be used and interpreted within their statistical framework in

order to gain a holistic picture of the metabolomics data (Hendriks et al., 2011; Saccenti et al., 2014).

1.6.2. Metabolomics in veterinary science

The word "metabolome" first appeared in the literature in 1998 (Oliver et al., 1998) and since then the field has rapidly evolved with a large diversity of research including nutrition, toxicology, gene function, physiology, disease mechanisms and environmental science (Jones and Cheung, 2007; Wishart, 2019). Despite this diversity of research applications, there is limited metabolomics research within the field of veterinary and animal science (Jones and Cheung, 2007). In a systemic review by Tran et al. (2020) investigating the number of metabolomics studies in the development of spontaneous animal disease, the authors found a total of 38 in which most of them utilised MS or NMR techniques. Interestingly, the majority of the studies were in dogs (13 studies), cows (12 studies) and horses (5 studies) and focused on the digestive and reproductive systems. In addition to this data, the authors also noted that there were 45 studies related to food or nutrition, 23 related to reproduction, 29 related to toxicology or doping with the bulk of the studies linked to laboratory disease models, cell culture and induced experimental diseases (88 studies). There is an increasing interest in the metabolomics of skeletal muscle and meat in domestic animals, termed "MEATabolomics", which is particularly useful in identifying the metabolome profiles of meat quality traits such as colour, water holding capacity, pH, flavour and palatability (Muroya et al., 2020). This is also important in highlighting how factors such as species, genetics, breed, feeding, muscle type, post-mortem ageing, meat storage, processing and spoilage, affect meat quality through the distinguishable metabolite differences (Muroya et al., 2020).

1.6.3. Metabolomics studies in the cheetah

The majority of metabolomics studies have been conducted in domestic or companion animals, with limited studies in wild or captive animals. Only a handful of studies have been conducted using metabolomic methods in captive or free-roaming cheetahs to investigate metabolic properties (Koester et al., 2017; Tordiffe et al., 2017, 2016; Tordiffe and Mienie, 2019; Weiner et al., 2019). Weiner et al. (2019) used GC-MS to investigate hexose and pyruvate utilisation and fatty acid oxidation in the spermatozoa in three felid species (cheetah, clouded leopard and domestic cat). While Koester et al. (2017) identified a novel faecal biomarker, Immunoglobulin J, of early pregnancy in captive cheetahs using mass spectrometry – this protein marker was elevated in pregnant females within 4 weeks of mating. Both these studies were focused on improving the reproductive knowledge of cheetahs.

Tordiffe et al. (2016), investigated the serum fatty acid profile of free-ranging and captive cheetahs using GC-MS and found that there was a decreased concentration of monosaturated, polyunsaturated and total fatty acids in free-ranging cheetahs compared to their captive

counterparts. A year later, Tordiffe et al. (2017) used a similar approach to study the urinary organic acid metabolites in apparently healthy captive cheetahs whose diet mostly consisted of donkey meat supplemented with minerals and vitamins. A third of the most abundant organic acids detected were phenolic compounds associated with microbial fermentation of phenylalanine, tyrosine and glycine conjugates. Additionally, an unknown compound was identified that is believed to be metabolised from cadeverine, which is produced by bacterial decarboxylation of lysine. The increased lysine and the high number of phenolic compounds is potentially a result of the increased muscle meat intake in captivity. Lastly, Tordiffe and Mienie (2019) used an untargeted exploratory GC-MS and LC-MS approach to determine the serum and urine amino acid profiles of captive cheetahs. In serum, 38 amino acids were identified with glutamine, alanine, arginine and glycine having the highest concentrations. Similarly, 38 amino acids were identified in urine with arginine, glutamine, alanine and serine having the highest concentrations. Arginine, an essential amino acid in carnivores, is required for ammonia detoxification in the urea cycle as well as creatine and nitric oxide synthesis. Under normal dietary conditions, arginine deficiencies are unlikely with large felids generally maintaining large arginine or ornithine reserves to deal with the acute ammonia loads resulting from protein rich diets. The latter three studies provide baseline data on serum and urine metabolites, which emphasises the benefit of taking a systems biology approach to identify a broad range of metabolites in a number of different metabolic pathways. Additionally, it highlights the role of diet in the whole body metabolism of both captive and free-ranging cheetahs. This is particularly important in captive cheetahs that are fed primarily muscle meat diets high in protein, with the metabolic effect of these diets requiring further investigation.

It is clear that the effect of the captive diet has been a growing area of research in cheetahs over the past 20 years and has been suggested as a contributor to the high prevalence of gastrointestinal diseases seen in this population. Glycine, as the most abundant amino acid in collagen, is required in substantial amounts in the cheetah, yet it is likely deficient in the captive diet. Therefore, understanding the metabolic role of collagen and glycine in cheetahs may provide crucial information on the effects of the captive diet. The use of metabolomics techniques, which are becoming increasingly popular in veterinary and nutritional studies, may be a useful tool to investigate these effects. This approach has not previously been used to investigate the effect of diet in captive cheetahs or any other exotic felid.

1.7. Aims of the study

The aim of the study was to investigate the metabolic differences between glycine supplemented and non-supplemented captive cheetahs in a randomised cross-over study design. This was achieved through haematology and serum biochemistry analyses as well as an untargeted metabolomics approach using NMR analysis on the urine and serum samples in 10 captive cheetahs.

CHAPTER TWO

2. STUDY DESIGN AND METHODOLOGY

2.1. Introduction

Randomised cross-over controlled trials are designed to allow the individuals within the study to experience one or more treatments in a randomised order, and are considered the strongest study design in biomedicine and pharmacology (Elbourne et al., 2002). There are a number of strengths for this design, namely the ability of individuals to act as their own controls which limits the amount of inter-group variation and allows for a smaller sample size to be used (Elbourne et al., 2002). Randomised controlled trials are frequently used with human participants – a systematic review found that of the 519 randomised trials published in December 2000, only 22% were cross-over designs, of which 70% included a washout period (Mills et al., 2009). Washout periods reduce the risk of a potential carryover effect between treatments, whereby an effect of one intervention is carried over to the next intervention (Elbourne et al., 2002). In terms of dietary intervention controlled trials, the limitations are largely linked to the behaviour of humans – high dropout rates, poor adherence to interventions, incorrect reporting, and limited generalisability to other population groups (Hébert et al., 2016). These limitations would not necessarily apply to diet interventions in captive or domestic animals. However, intervention study designs have rarely been used in captive animals, including cheetahs.

The use of metabolomics techniques (such as NMR, GC-MS) provides valuable information about what is happening within an individual, in response to external or environmental stimuli, and is becoming a popular tool in a variety of scientific fields (Wishart, 2019). Nutritional metabolomics is the integration of metabolic profiling and nutrition in complex biosystems with the goal of characterising the endogenous metabolome and the food metabolome, the latter including the metabolites derived from food consumption and their subsequent metabolism in the body (Guasch-Ferre et al., 2018). Compared to plasma and serum, urine contains a higher concentration of non-metabolite and non-nutrient compounds that result from food phytochemicals and other chemicals making it an acute marker of frequently consumed foods (Guasch-Ferre et al., 2018). Additionally, analysis of faecal and urine metabolic composition reflects the status of the gut microbiome, which is largely affected by diet, and bridges the gap between the microbiome and the health of the host (Jain et al., 2019). These nutritional metabolomics studies are becoming increasingly popular in human populations through observational or intervention studies, but are less frequently used in exotic or domestic animal populations.

Therefore, the use of a randomized cross-over design in combination with metabolomics techniques and conventional serum and haematology analysis, may be a useful tool to investigate metabolic changes in response to diet in captive cheetahs. The aims of this chapter are to:

- describe the study design and glycine diet intervention of 10 captive cheetahs;
- describe the sample collection, body measurements, serum biochemistry and haematology methods;
- describe NMR analysis, data analysis and statistical analysis workflow for the urine and serum samples.

2.2. Ethical approval

Ethical approval for this study was obtained from the Animal Research Ethics Committee of the Faculty of Veterinary Science, University of Pretoria (reference number: REC231-19) and the Animal Research Ethics Committee of the University of the Western Cape (reference number: AR20/3/4). This study was conducted under the standing Threatened or Protected Species (TOPS) permit for the Faculty of the Veterinary Science, University of Pretoria (reference number: S02559). A Department of Agriculture, Forestry's and Fisheries Section 20 permit was also granted for the undertaking of this research (reference number: 12/11/1/7).

2.3. Study design and feeding intervention

2.3.1. Overview of the study design

The study was designed as a randomised cross over dietary intervention on 10 captive cheetahs (*Acinonyx jubatus)*. The research venue was located at Cango Wildlife Ranch, Oudtshoorn, Western Cape, South Africa and took place during the months of August to November 2020. Ten apparently healthy adult male and female cheetahs between the ages of 2 and 5 years of age were included in the study group (Supplementary Table S7). To ensure the cheetahs had the same baseline diets, all animals were habituated to the control horse muscle minced meat (referred to as "horse meat" hereafter) and vitamin-supplemented diet for three weeks before randomly being assigned to a control or intervention group. The detail of the diets will be discussed later, but briefly: the control group was fed horse meat with a basic vitamin and mineral supplement (Panthera supplement, WildCat Nutrition, Pretoria, South Africa) and the intervention group was fed the same diet, but with the addition of 30g glycine powder per 1 kg meat fed (Glycine supplement, WildCat Nutrition, Pretoria, South Africa). The assigned cheetahs were fed the control or glycine supplemented diet for four weeks. This was followed by a two-week washout period where all animals received the control diet, thereafter the groups were crossed over to receive the opposite diet for a further four weeks. Samples were collected on three occasions – at baseline (after the habituation), after the first intervention and after the second intervention. An overview of the study design is shown in Figure 3.

2.3.2. Study animals and setting

The study included five male and five female cheetahs housed at Cango Wildlife Ranch in Oudtshoorn, Western Cape, South Africa. The males and females had an average age of 4.2 and 3.2 years, respectively, with an age range of 2 to 5 years old (Supplementary Table S7). At the time of the study, all animals were healthy with no clinical signs of gastritis or renal failure. The animals were housed in medium to large enclosures (400 m^2 to 1350 m^2) away from the public in a rural setting. All the females were housed individually. Two males were housed individually, two males were housed together and a third male was housed with his brother who was not included in this study. It is common for male siblings to remain housed together in captivity as they would form coalitions in the wild (Hayward et al., 2006; Woc Colburn et al., 2018). None of the female cheetahs had cubs prior to the study. The study took place towards the end of winter and progressed into spring, with the average maximum temperature ranging from 19°C (during the habituation phase), to 25°C (during the first intervention phase) and 32°C (during the second intervention phase).

2.3.3. Diet before the study

Prior to the start of the study, all animals received a varied diet containing 1.3 to 1.8 kg horse or donkey muscle meat with 100 g organ mince and 100 g shredded skin six days a week, with one fasting day in the week. Additionally, they received bones with meat once a week and a vitamin and mineral predator supplement, calcium mixture and iodated salt on alternating days. This diet combination was implemented for at least 6 months prior to the start of the study and likely in excess of 2 years for the older cheetahs in the study. Therefore, the cheetahs received a diet with moderate to high collagen content (and therefore glycine content) for an extended period of time which may have long lasting effects. In order to standardise the diet across the 10 cheetahs, a three week habituation period was implemented prior to the first baseline sample collection (T1).

2.3.4. Habituation period

All animals were fed the control diet during the three week habituation period. The standardisation was implemented to control the glycine intake of the cheetahs because glycine is found in high concentrations in collagen (in skin and bones), but is relatively low in meat. Therefore, a meat only diet was fed during the habituation and control phase.

During the habituation period, cheetahs received between 1.5 and 2.2 kg horse meat with 10 g of vitamin and mineral supplement (Panthera supplement, WildCat Nutrition, Pretoria, South Africa) for every 1 kg meat fed. The supplement was sprinkled over and rubbed into the meat. The weekly feeding frequency remained the same, which included one fasting day, resulting in a total of 18 feeding days. Food consumption was monitored and all food was consumed. Baseline data and samples were collected after the three week habituation period.

2.3.5. Trial period

Following the first sample collection (T1), cheetahs were randomly assigned to a Control (no glycine) or Intervention group (glycine supplemented). Due to a logistical matter, the groups were not evenly split. During the first phase, six cheetahs (two males, four females) were assigned to the control diet, whereas only four cheetahs (three males, one female) where assigned to the glycine supplemented diet. Those on the control diet simply continued with the same diet that was implemented during the habituation period. The cheetahs in the intervention group were fed the same horse meat with the vitamin and mineral supplement and an additional 30 g of glycine powder per 1 kg meat which was sprinkled and mixed into the meat. Cheetahs remained on these diets for four weeks, amounting to 25 feeding days, thereafter the second sample collection occurred (T2).

All animals then underwent a two-week washout period where they were fed the control diet to negate any effects caused by the glycine supplementation and return to baseline. The groups were then crossed-over and fed the corresponding diet for another four weeks with a total of 25 feeding days. Therefore, the six cheetahs previously on the control diet received the glycine supplemented diet, and the four cheetahs previously on the glycine supplemented diet received the control diet (without glycine). The third and final sample collection (T3) took place after the four weeks.

Throughout the study, the standard feeding procedures of Cango Wildlife Ranch were followed, particularly the time of day when food was provided, number of days and the amount of meat provided. The standard feeding procedures were determined by the appropriate staff based on the cheetah's feeding history, appetite and behavioural response to the food. The nutritional details of the horse meat (from literature), vitamin and mineral supplement and the glycine supplement are reported in Table 1.

2.4. Sample collection procedure

2.4.1. Immobilisation

Samples were collected over two days on three occasions – at baseline, after the first intervention and after the second intervention. The cheetahs were immobilised at each sampling event via intramuscular hand injection with 30 μg/kg medetomidine hydrochloride (10 mg/ml, Medetomidine, Kyron Laboratories Pty LTD, South Africa) in combination with 1 mg/kg zolazepam/tiletamine (100 mg/ml, Zoletil ®, Virbac, South Africa). Once sedated, the cheetahs were transported to the medical centre and maintained under anaesthesia with 1-2% isoflurane in oxygen for the duration of the collection period which lasted approximately one hour. Throughout the sedation period the standard anaesthetic and cardiovascular parameters of the cheetahs were monitored. Once all the samples were collected, the sedation was reversed with 5 mg intramuscular atipamezole (5 mg/ml, Antisedan®, Pfizer, South Africa). Recoveries took between 15 to 20 min. Cheetahs were monitored regularly throughout the day and the following day.

Table 1. Nutrient breakdown of horse muscle meat, vitamin and mineral supplement and glycine supplement

Nutrient	Horse muscle meat (per 100g) [a]	Vitamin and Mineral (per 10g) [b]	Glycine (per 30g) [b]
Moisture (g)	70.9		
Protein (g)	19.8		
Lipids (g)	6.63		
Ash (g)	0.98		
Cholesterol (mg)	61		
Energy (kcal)	140		
SCFA (g)	2.0		
Monounsaturated FA (g)	2.7		
Polyunsaturated FA (g)	1.1		
Linoleic acid (mg)	678		
α-linolenic acid (mg)	318		
20:2 n-6 (mg)	19		
20:3 n-6 (mg)	14		
Arachidonic acid (mg)	58		
Ammonia (g)	0.26		
Essential amino acids (g)			
Histidine	0.90		
Isoleucine	0.91		
Leucine	1.52		
Lysine	1.57		
Methionine	0.48		
Phenylalanine	0.82		
Threonine	0.84		
Tryptophan	0.15		
Tyrosine	0.67		
Valine	0.96		
Non-essential/ Conditionally essential amino acids (g)			
Alanine	1.18		
Arginine	1.16		
Aspartic acid	1.77		
Cystine	0.20		
Glutamic acid	2.83		
Glycine	1.04		30
Proline	0.89		
Serine	0.69		
Hydroxyproline (g)	0.15		
Total collagen (g)	1.17		
Soluble collagen (%)	10.2		
Purine bases (mg)			
Adenine	18.29		
Guanine	8.23		
Xanthine	9.01		
Hypoxanthine	74.03		

Mineral (mg)			
Sodium	74.2		
Potassium	331.0		
Magnesium	28.9		
Calcium	3.8	3000	
Phosphorous	231.0	670	
Iron	3.9		
Zinc	3.7		
Copper	0.2	4.0	
Manganese		1.5	
Sodium chloride		340	
Vitamins			
Vitamin A (IU)		5000	
Thiamin (B_1) (mg)	0.043	5.0	
Riboflavin (B_2) (mg)	0.18	2.5	
Niacin (B_3) (mg)	5.54		
Pantothenic acid (B_5) (mg)		10	
Pyridoxine (B_6) (mg)	0.64		
Biotin (B_7) (µg)		100	
Folic acid (B_9) (µg)		800	
Vitamin B_{12} (µg)	2.08		
Vitamin C (mg)		125	
Vitamin D_3 (IU)		100	
Vitamin E (mg)		50	

[a] Values obtained from Badiani et al. (1997); [b] values obtained from WildCat Nutrition, Pretoria, South Africa. SCFA, short chain fatty acids

2.4.2. Sample collection and storage

At the medical centre, the cheetahs were weighed, and body length and height were measured while the cheetah was in lateral recumbency. Length was measured from the occiput at the caudal edge of the skull to the base of the tail and height was measured from the dorsal rim of the scapula to the metacarpal pad. Length and height were only measured during the first sampling (T1). The size index of each cheetah was calculated by multiplying the body length with the shoulder height. The size index was divided by weight to determine the body mass index (BMI) of the cheetahs (Kirberger and Tordiffe, 2016).

Blood was collected from the jugular vein, using an 18G needle and 20 ml syringe, and immediately transferred to an EDTA and serum tube. The fresh blood was handled with care to avoid haemolysis of the red blood cells. The whole blood in the EDTA tube was used for haematological analysis on site (Abaxis Vetscan HM5 haematology system). After analysis, whole blood was stored at -20°C. The blood in the serum tube was allowed to clot for 30 min at room temperature and subsequently centrifuged for 10 min at 6000 rpm. The supernatant was transferred to a new microtube and stored at -20°C.

Urine was collected via urethral catheterisation using a sterile 6FG 120 mm feeding tube, after which it was centrifuged for 10 min at 6000 rpm to remove any cellular debris. The urine was then transferred to a microtube and stored at -20°C. The specific gravity of the urine was determined using a handheld refractometer: a drop of urine was placed on the glass cover and the specific gravity visually determined using an internal calibrated grid. The urine of the cheetah in general is more concentrated compared to humans and, therefore, values of between 1065 to 1080 au would indicate normal functioning kidneys. Specific gravity was evaluated at each sample collection time point (T1, T2 and T3) to ensure that all animals had normal kidney function to continue with the study. The urine specific gravity was appropriate for each cheetah with an average of 1069 ± 6 au.

Serum, whole blood and urine were transported on dry ice (-60°C) to the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria and stored at -80°C. Serum biochemistry analysis was conducted in the analytical laboratory at the University of Pretoria, while the remaining serum, urine and skeletal muscle samples were transported on dry ice to the Centre for Human Metabolomics, North West University, where they were stored at -80°C until NMR analysis.

2.5. Blood analyses

2.5.1. Serum biochemistry and haematology

Whole blood was analysed on site at Cango Wildlife Ranch using the Abaxis Vetscan HM5 haematology system set to the cat species setting. The white blood cell (WBC) and red blood cell (RBC) count, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet count and percentage, mean platelet volume (MPV) and platelet distribution width (PDW) were determined.

Routine serum biochemistry analyses were performed in the Clinical Pathology laboratory, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria. The concentration of the following were determined using the Cobas Integra® 400 plus analyser (Roche, Randburg, South Africa): Total serum protein (TSP), albumin (Alb), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine, phosphate, triglycerides and total calcium. This is a high throughput, fully automated, software controlled system that uses absorbance spectrophotometry, fluorescence polarisation and electrolyte analysis. Sodium, potassium and chloride were measured using the Rapidpoint® 500 System (Siemens Ltd, Isando, South Africa) specific electrolyte assay cartridges.

2.5.2. Statistical analysis

Statistical calculations for blood analyses were performed using GraphPad Prism 5 version 5.03 (GraphPad Software, CA, USA). Values are expressed as mean ± standard deviation (SD) and

normality was checked within diet groups using the Shapiro-Wilks normality test. As the study design split the animals into Group 1 with six animals and Group 2 with four animals (Figure 3), a non-parametric Mann-Whitney U test was performed to identify if there was any difference between the split groups for all measured variables. There was no difference between groups, therefore, groups were combined under each diet, i.e. a combined baseline, control (no glycine diet) and glycine supplemented diet. The groups were combined in all further analysis. A repeated measures one-way ANOVA with a Tukey post hoc test was used to compare the three groups. Significance was set at $p < 0.05$.

2.6. NMR analysis

At the conclusion of the feeding intervention and sample collection period, all samples were transported on dry ice to the Centre for Human Metabolomics, North West University, for NMR analysis. An untargeted whole metabolome ^{1}H-NMR approach was utilised to identify and quantify the metabolites present in the urine and serum samples of 10 captive cheetahs at baseline, on a control (no glycine) diet and on a glycine supplemented diet.

2.6.1. Urine sample preparation

A 1 mL urine aliquot was added to a micro tube and centrifuged for 5 min at 12 000 x g. Thereafter, 540 µL was transferred to another micro tube and mixed with 60 µL of NMR buffer solution (9:1). The NMR buffer solution consisted of 1.5 M potassium phosphate buffer (pH 7.4) in deuterium oxide and 5.8 mM trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP; Sigma-Aldrich, St. Louis, Missouri, USA) with a trace amount of sodium azide included to prevent bacterial growth in the sample. This was followed by centrifugation for 5 min at 12 000 x g. A final volume of 540 µL was transferred to a 5 mm NMR tube for metabolomics analysis.

2.6.2. Serum sample preparation

Samples were filtered using Amicon Ultra 2 mL centrifugal units with 10 kDa membrane filters (Merck; UFC201024). Prior to use, each centrifugal filter unit was pre-rinsed with dH_20 and centrifuged at 4500 x g for 10 min to remove trace amounts of glycerol and glycerine from membrane filters, which can interfere with NMR signals. 800 µL of serum was then filtered and centrifuged at 4500 x g for 30 min. 540 µL of each filtered sample and 60uL buffer solution (same as urine) was then added to a micro centrifuge tube and mixed. The sample mixture was centrifuged at 12 000 x g for 5 min. A final volume of 540 µL was transferred to a 5 mm NMR tube for metabolomics analyses.

2.6.3. System and experimental settings

The same settings were used for urine and serum. A 500 MHz Bruker Avance III HD NMR spectrometer equipped with a triple-resonance inverse (TXI) ^{1}H {^{15}N, ^{13}C} probe head and x, y, z

gradient coils was used to analyse the samples. ^{1}H spectra were acquired as 128 transients in 32K data points with a spectral width of 10504 Hz and acquisition time of 3.12 s. The receiver gain was set to 90.5. The sample temperature was maintained at 300K and the H_2O resonance was pre-saturated by single-frequency irradiation during a relaxation delay of 4 s, with a 90° excitation pulse of 8 µs. Shimming of the sample was performed automatically on the deuterium signal. Fourier transformation and phase and baseline correction were done automatically. Software used for NMR processing was Bruker Topspin (version 3.5).

2.7. Data analysis

The raw Bruker Topspin spectral data files were obtained and spectra analysis was performed using R programming software (R, version 4.0.4) and RStudio integrative environment (RStudio, version 1.4.1106, MA, USA). The Automatic Statistical Identification in Complex Spectra (ASICS; version 2.6.1, Bioconductor, NY, USA) R package was used to identify and quantify metabolites in the complex spectra. This is described in detail by Lefort et al. (2019) and Tardivel et al. (2017). The following sections are a summary of the various data analysis steps.

2.7.1. Data processing

Briefly, the raw Bruker spectral files were imported into RStudio and the complex spectrum underwent several pre-processing steps. Firstly, baseline distortions caused by instrument instability were corrected by constructing a baseline and removing it from the spectrum. Secondly, a speaq algorithm was used to align the spectra against an automatically detected reference spectrum in order to remove any metabolite peak position change due to temperature or pH variations. Thirdly, unwanted regions in the spectrum corresponding to water (4.5 to 5.1 ppm) and urea (5.5 to 6.5 ppm) were removed as their wide range can interfere with the detection of less concentrated molecules. The final pre-processing step was normalisation which minimises the systematic variations due to differences in sample dilutions, therefore making samples comparable - the Probabilistic Quotient Normalisation (PQN) method was used for this dataset (Dieterle et al., 2006). The PQN method assumes that changes in concentrations of a single analyte only influence parts of the spectra and, therefore, the intensity of a majority (but not all) of the signals is a function of dilution. PQN appears to be a more robust and consistent method than normalising to a constant sum. This method of normalisation was selected over correcting to creatinine concentration because creatinine has been shown to be highly variable in cheetahs, and this more holistic method of normalising may provide a better representation of the NMR spectrum (Ryan et al., 2011; Tordiffe et al., 2017).

The ASICS package provides a pre-processed library of the spectra of pure compounds which was used as a reference to identify and quantify metabolites found in the complex spectra of interest. The list of library compounds is included in Supplementary Table S1. Several steps were required

to adapt the library to the spectra of interest, such as noise thresholding, removal of library metabolites that were not found in the complex mixture spectra and the alignment of the library spectra with the complex mixture spectra.

2.7.2. Metabolite identification and quantification

The identification of metabolites in the complex sample mixture relies on various linear and warping functions. This provides reliable identification by controlling two possible sources of error: identifying a metabolite that is not present in the complex mixture (false positive) or not identifying a metabolite that is present (false negative). A least squares method was used to quantify the identified metabolites. This method did not allow identification of metabolites at an absolute concentration below 1 µM, as the signal to noise ratio was too low, as well as metabolites at an absolute concentration above 1 M which resulted in broadening and overlapping of the NMR signals.

This process identified metabolites and provided an estimated relative concentration which is a function of the complex mixture, reference library and number of protons for each selected metabolite. This relative concentration or quantification allows comparison between samples, but it is not an absolute concentration. The list of identified metabolites and their relative quantifications for each cheetah was exported from RStudio as an Excel workbook.

2.8. Statistical analysis

All statistical analysis for NMR data was conducted using MetaboAnalyst version 5.0 (https://www.metaboanalyst.ca) (Chong et al., 2019; Pang et al., 2021). JMP Pro version 16.1.0 (SAS Institute, North Carolina, USA) was used to create the figures. Data are expressed as mean ± standard deviation where appropriate and significance was set at $p<0.05$.

MetaboAnalyst is a comprehensive tool that offers a variety of univariate and multivariate tests specifically designed for large datasets, such as in metabolomics studies, but limitations within this application do exist. In particular, MetaboAnalyst has very limited analyses for paired data of multiple groups, with its strength being the analyses of unpaired groups or two paired groups. The current study has a cross-over design which creates three paired groups at baseline, on the control diet and on the glycine supplemented diet. In order to obtain a more holistic statistical overview, multiple univariate and multivariate tests were utilised after the data normalisation steps. This more "thoughtful" approach, as described by Wasserstein et al. (2019) prioritises the scientific context and makes use of multiple approaches to solve a problem, which is especially relevant considering the exploratory nature of this study. The various approaches are described below, which combined, give rise to the notable potentially important metabolites of interest. This process is summarised in Figure 3.

2.8.1. Pre-analysis check of groups

The current study design included a total sample of 10 cheetahs which were divided into 2 uneven groups of n=6 (Group 1) and n=4 (Group 2) which is displayed in Figure 3. These groups experienced different lengths of time on the control (or glycine deficient diet) as well as either one (Group 1) or two (Group 2) dietary changes during the study. Group 1 experienced a total of 9 consecutive weeks on the control diet, while Group 2 experienced 6 consecutive weeks on the control diet as the glycine intervention occurred immediately after the habituation period in the latter group. Therefore, these groups may have different levels of glycine deficiency with Group 1 potentially being a better representation of glycine deficiency before experiencing the glycine intervention. As a result, it was necessary to first check the urine and serum metabolite analysis for each group before examining the combined effect. The steps described below were conducted for both the n=6 group and the combined n=10 group. The details and results of the n=6 analysis are described in Appendix 2. After the analysis it was deemed suitable to use the combined n=10 groups as it produced more consistent data and this approach was utilised in Chapters 4 to 6.

2.8.2. Data cleaning and scaling

Metabolites that were present in less than 50% of the samples in all three groups (i.e. baseline, control and glycine supplemented) were removed in order to reduce the noise or effect of infrequently observed metabolites, across all samples, in the subsequent analyses. After cleaning, the metabolite concentration list was imported into MetaboAnalyst 5.0. The data underwent various steps prior to analysis, including an integrity check, replacing zero values by 20% of the lowest value, data filtering based on interquartile range, which removed approximately 10% of outlying values and auto scaling normalisation which makes the metabolites more comparable by scaling to the sample mean and standard deviation. As there was a wide range of concentrations between metabolites, these steps were necessary to ensure that all the values were equally weighted especially for multivariate analysis. It makes use of the following equation to calculate the scaled value for a single metabolite of a sample: scaled value = $(X_2 - X_1)/SD_1$ where X_2 is an individual sample metabolite concentration, X_1 is the metabolite mean for all samples, SD_1 is the metabolite standard deviation for all samples.

2.8.3. Univariate analysis

A one-way repeated measures ANOVA was performed to identify differences in metabolites between the baseline, control and glycine supplemented groups. False Discovery Rate (FDR) was used to correct for the multiple univariate comparisons and protect against false positive results. A post hoc analysis was not possible using MetaboAnalyst. Thus, the normalised and scaled data of the significantly identified metabolites from MetaboAnalyst was imported into JMP to identify where

the difference was between groups using a Tukey-Kramer HSD post hoc analysis adjusting for multiple comparisons.

2.8.4. Multivariate analysis

Principle component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) was performed on the normalised data in MetaboAnalyst. PCA is an unsupervised data reduction method which does not take class labels (diet) into account when explaining the variance within the data, while PLS-DA is a supervised data reduction method which takes the class labels into account when creating predictive models for the different classes. Importantly, both these methods allow the reduction of large or multi-dimensional datasets with many variables into simple one-dimensional datasets that retains the most important components contributing to the variation. This allows you to work with compressed datasets without losing the crux of the original data (Saccenti et al., 2014) For PLS-DA, the leave-one-out cross validation method was used for five components in which the cross validated sum of the squares (Q2) was an estimate of the predictive ability of the model. Loading scores, which indicate the influence of each metabolite on the variation seen in the data, were recorded for PCA. The important measures for PLS-DA, namely the variable importance in projection (VIP) and the weighted sum of absolute coefficient of regression for each diet, were recorded for each metabolite. All three recorded measures for PCA and PLS-DA provide an indication of the metabolites that are contributing the most to the variation or separation seen in the analyses and can be used as a tool to highlight metabolites of interest. All metabolites were ranked from highest to lowest influence for PC 1 and PC 2 for the PCA loading scores and PLS-DA VIP sores and for each diet of the PLS-DA coefficient of regression.

2.8.5. Identifying metabolites of interest

The metabolites identified with the one-way ANOVA and the top 20% of metabolites with the greatest influence under each parameter of the PCA (loading scores) and PLS-DA (VIP and coefficient of regression) analysis were highlighted. These metabolites were further reduced to those that were present in four or more analyses out of a potential of 8. This ultimately created a list of potentially important urinary and serum metabolites in captive cheetahs on a baseline, control and glycine supplemented diet.

An overview of the study methodology is shown in Figure 3. The results obtained will be discussed and structured as follows: Chapter 3 will focus on the body measurements, serum biochemistry and haematology; Chapter 4 will focus on the urine metabolite; Chapter 5 will focus on the serum metabolite; and Chapter 6 will provide a general discussion and conclusion.

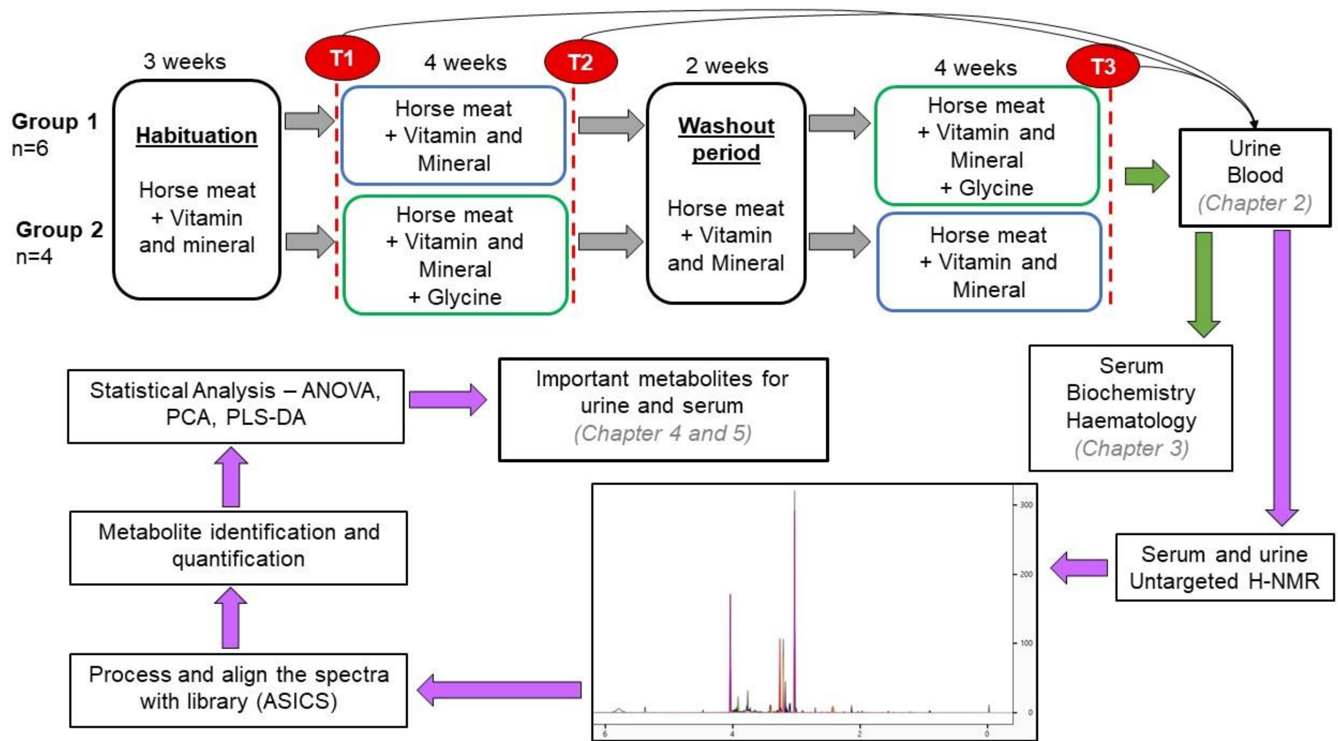

Figure 3. Overview of the study methodology including study design, feeding intervention, data analysis and statistical analysis.

CHAPTER THREE

3. BODY MEASUREMENTS AND BLOOD DATA

3.1. Introduction

The morphology and physical condition were extensively investigated in wild Namibian cheetahs, in which there was notable sexual dimorphism. Adult males weighed 45.6 ± 6.0 kg and females 37.2 ± 5.1 kg, with the adult body mass only being reached between four to eight years of age (Marker and Dickman, 2003). The average body length and shoulder height of males and females were 1.02 m and 0.77 m, and 0.98 m and 0.74 m, respectively. In a captive Namibian population, the average body weight of males and females were 40.1 kg and 36.4 kg, respectively, while the body length and shoulder height were 0.99 m and 0.73 m (for males), and 0.96 m and 0.69 m (for females) (Kirberger and Tordiffe, 2016). Kirberger and Tordiffe (2016) used those three measurements to establish a size index and body mass index (BMI) for cheetahs. There is variation in the size and dimensions of cheetahs depending on the population group, with captive cheetahs generally being smaller and lighter, as well as the methods of measurements (Marker and Dickman, 2003).

Haematology and serum biochemistry have been analysed in both captive and free-ranging cheetahs in several studies (Bechert et al., 2002; Caro et al., 1987; Depauw et al., 2012b; Hawkey and Hart, 1986; Hudson-Lamb et al., 2016). Of all these studies, two stand out, where Bechert et al. (2002) investigated the blood values of captive cheetahs fed a commercial or supplemented meat diet and Depauw et al. (2012) investigated the serum data of captive cheetahs fed a whole rabbit or supplemented beef diet.

Although diet has been investigated in a limited number of studies in captive cheetahs, the effect of glycine supplementation has not been investigated in this population specifically in regards to morphometric and blood parameters. Therefore, the aims of this chapter are to:

- Compare the feeding and body measurements between baseline, control and glycine supplemented groups.
- Compare the haematology data between baseline, control and glycine supplemented groups.
- Compare the serum biochemistry data between baseline, control and glycine supplemented groups.

3.2. Results

3.2.1. Diet and feeding

No adverse response to the control or glycine supplemented diet was reported. Glycine is sweet and highly palatable (McCarty et al., 2018) and all the food offered to the cheetahs was consumed. The amount of food provided to each animal remained fairly consistent throughout the study with three males, from Group 2, having a 200g meat increase as the study progressed (12.5% average increase). One male increased from the habituation period to the glycine supplemented diet, and the other two increased from the glycine supplemented diet to the control diet. The average consumption for males and females per dietary phase is reported in Table 2.

Table 2. Average daily amount of meat, vitamin and mineral supplement and glycine supplement for male (n = 5) and female (n = 5) cheetahs during the habituation, control and glycine intervention periods.

	Horse muscle meat (kg)	Vitamin and mineral supplement (g)	Glycine supplement (g)
Habituation (3 weeks)			
Male	1.7 ± 0.3	17.4 ± 2.5	0
Female	1.8 ± 0.3	18.0 ± 2.6	0
Control diet (4 weeks)			
Male	1.9 ± 0.2	18.6 ± 1.5	0
Female	1.8 ± 0.3	18.0 ± 2.6	0
Glycine diet (4 weeks)			
Male	1.8 ± 0.3	17.8 ± 2.6	53.4 ± 7.8
Female	1.8 ± 0.3	18.0 ± 2.6	54.0 ± 7.6

Data are expressed as mean ± SD.

3.2.2. Body measurements

The weight and body measurements of the study cheetahs are reported in Table 3. As the body parameters of cheetahs have been shown to be sexually dimorphic (Marker and Dickman, 2003), male and female data have been shown separately. However, statistical difference was only investigated between the different diets, and not sex, as groups were matched. The average weight at baseline and on the control diet were 40.2 ± 4.5 kg and 39.5 ± 4.0 kg, respectively. Weight significantly decreased to 38.8 ± 4.4 kg from baseline on the glycine supplemented diet. At baseline, the BMI of the cheetahs was 52.2 ± 3.6 kg/m^2 which was similar to the control diet at 51.2 ± 2.9 kg/m^2. The glycine diet had a significantly lower BMI of 50.3 ± 3.5 kg/m^2 compared to the baseline. All the parameters fall within the ranges reported by Kirberger and Tordiffe (2016).

Table 3. Body weight and measurements of male (n=5) and female (n=5) captive cheetahs at baseline, control and glycine diet.

	Baseline		Control Diet		Glycine Diet		Reference range
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
Weight (kg)	40.2*	4.5	39.5	4.0	38.8*	4.4	
Female	37.5	2.6	36.8	2.3	36.5	2.3	*30.9-44.1*
Male	43.0	4.6	42.2	3.6	41.1	4.9	*33.6-50.6*
Body Length (m)							
Female	1.00	0.04	—	—	—	—	*0.92-1.01*
Male	1.03	0.06	—	—	—	—	*0.93-1.10*
Shoulder height (m)							
Female	0.74	0.02	—	—	—	—	*0.64-0.73*
Male	0.77	0.02	—	—	—	—	*0.68-0.77*
Size index (m^2)							
Female	0.74	0.04	—	—	—	—	*0.61-0.72*
Male	0.80	0.06	—	—	—	—	*0.63-0.84*
BMI (kg/m^2)	52.2*	3.6	51.2	2.9	50.3*	3.5	
Female	50.6	1.9	49.7	1.1	49.3	0.8	*45.9-66.4*
Male	53.7	4.4	52.7	3.5	51.3	5.0	*42.9-63.1*

** indicates p<0.05 between combined male and female Baseline and No Glycine groups; — indicates data was not measured; BMI, body mass index. Size index calculated as body length x shoulder height. BMI calculated as weight/size index. Reference range from Kirberger and Tordiffe (2016).*

3.2.3. Haematology

The whole blood haematology data are shown in Table 4. Neutrophils contributed the most to the count with 7.97 ± 2.45 $x10^9$/L (baseline), 8.49 ± 4.09 $x10^9$/L (no glycine control) and 8.62 ± 2.74 $x10^9$/L (with glycine). Eosinophils increased significantly from baseline (0.15 ± 0.07 $x10^9$/L) to the control diet (0.26 ± 0.08 $x10^9$/L) and the glycine diet (0.25 ± 0.09 $x10^9$/L). Similarly, the basophil count also increased from the baseline diet (0.01 ± 0.01 $x10^9$/L) to the control and glycine supplemented diets with 0.02 $x10^9$/L, albeit remaining the lowest count of the white blood cells.

The RBC count remained similar across the diets with 8.72 ± 0.45 $x10^{12}$/L at baseline, 8.54 ± 0.44 $x10^{12}$/L with the control diet and 8.33 ± 0.59 $x10^{12}$/L with the glycine diet. The various RBC parameters remained similar across the three diets – haemoglobin, haematocrit, MCV, MCH and RDW. The platelet count was 406 ± 124 $x10^9$/L (baseline), 396 ± 78 $x10^9$/L (no glycine diet) and 429 ± 89 $x10^9$/L (glycine diet). The platelet parameters, including mean platelet volume (MPV) and volume in the blood (PCT), remained similar across the diets. The platelet distribution width significantly increased from baseline (42.5 ± 0.3%) to the glycine supplemented diet (42.8 ± 0.5%), with the control diet at 42.6 ± 0.5%.

The reference haematology data are from Bechert et al. (2002) and Hawkey and Hart (1986) to provide an indicator of the range found in captive cheetahs. All the haematological data across the diets fall within the reference ranges provided in Table 4.

Table 4. Haematology data of 10 captive cheetahs at baseline, with the control and glycine diet.

	Baseline		Control Diet		Glycine Diet		Previous literature	
Parameters	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Bechert[a]**	**Hawkey[b]**
WBC (x10⁹/L)	11.12	3.24	11.30	4.19	11.31	2.81	*7.6-16.2*	*5.9-12.0*
Lymphocytes	2.44	1.51	1.90	0.47	1.88	0.31	*0.9-3.1*	*0.9-2.7*
Monocytes	0.55	0.28	0.63	0.37	0.54	0.30	*0.07-0.64*	*0-0.3*
Neutrophils	7.97	2.45	8.49	4.09	8.62	2.74	*5.4-12.9*	*5-9.4*
Eosinophils	0.15*#	0.07	0.26*	0.08	0.25#	0.09	*0.09-0.81*	*0.0-1.4*
Basophils	0.01*#	0.01	0.02*	0.00	0.02#	0.01	—	*0*
RBC (x10¹²/L)	8.72	0.45	8.54	0.44	8.33	0.59	*5.7-10.3*	*6-8.3*
Haemoglobin (g/L)	160.00	8.46	155.80	8.72	153.60	8.81	*102-195*	*116-166*
Haematocrit (%)	48.43	2.06	47.47	1.74	46.51	2.57	*29.3-58.7*	
MCV (fL)	55.60	1.71	55.60	1.71	55.90	1.73	*46.1-58.1*	*50-62*
MCH (pg)	18.38	0.87	18.23	0.69	18.46	0.50	*15.9-20.0*	*18.4-21.8*
MCHC (g/L)	330.60	13.90	327.70	7.39	330.30	6.07	*328-348*	*315-369*
RDWc (%)	19.59	0.50	19.80	0.49	19.48	0.35	—	—
RDWs (fL)	40.55	1.83	40.92	1.32	40.54	1.40	—	—
Platelets (x10⁹/L)	406.00	123.58	395.60	78.47	429.00	89.09	*243-475*	—
MPV (fL)	12.60	1.30	12.61	0.71	12.54	0.81	—	—
PCT (%)	0.50	0.14	0.50	0.09	0.53	0.08	—	—
PDWc (%)	42.53#	0.31	42.57	0.49	42.84#	0.50	—	—
PDWs (fL)	26.21	1.32	26.27	1.48	26.81	1.19	—	—

** indicates p<0.05 between Baseline and Control groups; # indicates p<0.05 between Baseline and Glycine groups; — indicates data not available. WBC, white blood cells; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width; MPV, mean platelet volume; PCT, platelet volume in blood; PDW, platelet distribution width. Reference range from Bechert et al. (2002) and Hawkey and Hart (1986).*

3.2.4. Serum biochemistry

The serum biochemistry data of the 10 captive cheetahs are shown in Table 5. The TSP and, therefore, the albumin concentration was significantly different from the baseline (TSP: 63.5 ± 2.8 g/L; albumin: 42.7 ± 1.8 g/L) to the control diet (TSP: 61.9 ± 3.4 g/L; albumin: 40.6 ± 2.1 g/L) and glycine diet (TSP: 60.9 ± 3.1 g/L; albumin: 40.5 ± 1.6 g/L). ALP, an enzyme predominantly synthesised in the liver, was significantly higher at baseline (14.5 ± 9.4 U/L) compared to the control diet (11.2 ± 6.7 U/L) and glycine supplemented diet (10.2 ± 5.7 U/L). Potassium, on the other hand, was higher in the control diet (4.2 ± 0.1 mmol/L) compared to the baseline (3.9 ± 0.3 mmol/L), whereas the glycine supplemented diet (4.1 ± 0.2 mmol/L) was similar to the baseline diet. The total calcium concentration (bound and free) was higher at baseline (2.66 ± 0.06 mmol/L) compared to the glycine supplemented diet (2.59 ± 0.05 mmol/L), with the control diet at 2.61 ± 0.09 mmol/L. ALT, urea, creatinine, sodium, phosphate, chloride and triglyceride concentrations remained constant across the three groups.

The serum reference data included are based on the research conducted by Bechert et al. (2002), Depauw et al.(2012) and Hudson-Lamb et al. (2016). Most of the parameters in Table 5, including those that were significantly different, fall within the indicated reference ranges, but ALP activity is noticeably lower in the current study compared to what was reported by Bechert et al. (2002).

Table 5. Serum data of 10 captive cheetahs at baseline, control diet and glycine diet.

	Baseline		Control Diet		Glycine Diet		Previous literature		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Bechert[a]**	**Hudson-Lamb[b]**	**Depauw[c]**
TSP (g/L)	63.54*#	2.84	61.87*	3.37	60.93#	3.14	*60-76*		*69 ±1*
Albumin (g/L)	42.65*#	1.81	40.56*	2.08	40.51#	1.59	*35-43*		*40 ±1*
Globulins (g/L)	20.91	2.13	21.31	2.76	20.44	2.56	*24-33*		*29 ±1*
ALT (U/L)	79.19	23.78	74.37	17.80	71.96	18.06	*66-198*		*69 ±13*
ALP (U/L)	14.50*#	9.44	11.22*	6.68	10.23#	5.65	*22-30*		
Urea (mmol/L)	15.12	3.15	14.29	2.23	14.73	2.38		*7.4-22.9*	*16.1 ±2.3*
Creatinine (µmol/L)	190.50	29.30	182.90	20.19	184.80	26.93	*176-389*	*114-276*	*180 ±23*
Na (mmol/L)	155.58	0.68	155.27	1.01	155.09	0.47		*148-160*	*157 ±1*
K (mmol/L)	3.97*	0.25	4.22*	0.11	4.07	0.17		*3.8-5.2*	*4.2 ±0.1*
Cl (mmol/L)	121.14	1.33	121.93	1.78	121.69	1.45		*116-129*	*122 ±2*
Phos (mmol/L)	1.65	0.23	1.66	0.24	1.72	0.28			*1.9 ±0.3*
Triglycerides (mmol/L)	0.53	0.12	0.54	0.12	0.47	0.12			*0.59 ±0.16*
Ca(T) (mmol/L)	2.66#	0.06	2.61	0.09	2.59#	0.05			*2.7 ±0.1*

** indicates p<0.05 between Baseline and No Glycine groups; # indicates p<0.05 between Baseline and Glycine groups. TSP, total serum protein; ALT, alanine aminotransferase; ALP, alkaline phosphatase; Na, sodium; K, potassium; Cl, chloride; Phos, phosphate; Ca(T), total calcium. Reference range from Bechert et al. (2002), Hudson-Lamb et al. (2016) and Depauw et al. (2012).*

3.3. Discussion

3.3.1. Body measurements and weight loss

There was a decrease in the average body weight of the cheetahs from baseline to the glycine supplemented diet, and this was likely due to a reduction in the body weight of the males as the females remained fairly consistent in weight. This change in weight also led to a corresponding decrease in BMI from the baseline to the glycine supplemented diet, which was calculated using the weight, length and shoulder height of each animal. Although the weight of the animals decreased, they were still well within a healthy range, such as what was provided by Kirberger and Tordiffe (2016). As reported in Table 2, the amount of meat consumed by each animal remained constant throughout the study, with a marginal increase during the glycine phase with ± 100 g meat – this was due to an increase in the food consumption of one male in Group 2 after the habituation period. The other change in the amount of meat consumed was for two males, also in Group 2, who

increased their intake by 200 g each as they moved from the glycine supplemented diet to the control diet. This is shown by the slightly higher amount of muscle meat consumed during the control diet phase. This increase was determined by the designated carers who deemed it necessary due to apparent hunger based on the cheetah's behaviour. Therefore, the weight loss is not due to a decrease in food intake, as the amount remained constant or increased slightly for three individuals.

The study started in August, which is the last month of winter in the Southern hemisphere, and progressed through to early November, marking the end of spring. As expected, there was a large average daily temperature increase between the habituation phase (12 ± 4 °C), the first diet phase (16 ± 4 °C) and the second diet phase (22 ± 5 °C). Colder ambient temperatures promote thermogenesis in which organisms activate adaptive metabolic processes to maintain core body temperature, therefore increasing daily energy expenditure (Hansen et al., 2010). Therefore, the increase in environmental temperature is unlikely to account for the weight loss of the cheetahs. Historically cheetahs have occupied a wide variety of habitats including dry forests to arid deserts and would undoubtedly be adapted to the hot and humid conditions of the area (Durant et al., 2015). It is likely that any of these acute changes (in particular in response to the changed diet) would have occurred during the habituation phase before the start of the study or would be controlled for by the split grouping of the animals.

A potential contributor to the weight loss is the study diet itself, which would be sub-optimal. Prior to the study, the animals received a combination of organ mince, skin and bones which would provide them with a greater array of nutrients more aligned to their carnivorous diet in the wild. However, in order to investigate the effects of the glycine alone, it was necessary to remove all other potential sources of glycine, such as the collagen of bones and skin (Li and Wu, 2018). While body weight appeared to decrease from the baseline to the control diet, it was not significantly different, indicating that the glycine supplemented diet potentially played a larger role in weight loss. Interestingly, when the group of four and six cheetahs were looked at separately there was still a significant reduction in weight from the baseline to glycine supplemented diet. In healthy humans supplemented with 1 mmol glycine/kg body weight, the concentration of plasma glycine increased 4 fold within 40 min and remained elevated for 2 hours, indicating rapid absorption of glycine (Gannon et al., 2002). Glycine also constantly stimulated the release of glucagon (which increases blood glucose), but in contrast, when glycine was ingested with glucose, this reduced the plasma glucose concentration by half. The authors speculated that the oral ingestion of glycine stimulates the secretion of a gut hormone that works with insulin to reduce glucose circulation and inhibits the effect of glucagon despite the increased concentration (Gannon et al., 2002). In sucrose fed rats (which promotes intra-abdominal fat accumulation), glycine supplementation reduced the amount of intra-abdominal fat, blood pressure, adipocyte size and the concentration of various non-esterified fatty acids (e.g. palmitic acid, arachidonic acid) returned to control levels (El Hafidi et al.,

2004). This forms part of the increasing evidence base of the beneficial glycine supplementation effects in the potential treatment of obesity and type 2 diabetes (Wang et al., 2013). Glucose and insulin concentrations were not measured in the serum of the captive cheetahs using this particular method, but glucose was identified with the NMR analysis. The effect of glycine on this aspect of metabolism may have contributed to their weight loss through the loss of fat and increased β-oxidation or other changes in carbohydrate and fat metabolism (El Hafidi et al., 2004).

3.3.2. Haematology

Although not significant, there was an increase in the total WBC count from the baseline diet to the control and glycine supplemented diet. However, the increase in eosinophils and basophils were significantly higher between diets. The WBC measured in Table 4 forms part of the innate immune system and provides the front line, rapid response to foreign microbes – the innate and acquired immune system are dependent on the availability of certain amino acids for the biosynthesis of proteins and polypeptides (Li et al., 2007). Therefore, dietary protein deficiencies compromise the immune system and its ability to function effectively (Li et al., 2007). Glycine is an effective anti-inflammatory and immunomodulatory agent, although the mechanisms of its action is not completely understood (Zhong et al., 2003). A potential mechanism is the activation of the glycine receptor (GlyR), which is a glycine gated chloride channel found in a variety of neuronal, inflammatory and immune response cells such as macrophages, monocytes and neutrophils (Zhong et al., 2003). The binding of glycine acts as an inhibitory neurotransmitter leading to the influx of chloride into the cells, which counters the effect of any excitatory neurotransmitter and also blocks the influx of intracellular calcium. This prevention of intracellular calcium suppresses the inflammatory response of these cells, and therefore, glycine plays a regulatory role in the production of cytokines and inflammatory mediators, enzyme activation, cell proliferation and cell death (Zhong et al., 2003).

However, the seemingly slight increase in WBC in the control and glycine supplemented diet, largely driven by the increase in the granulocytes (neutrophils, basophils and eosinophils), is likely due to the sub-optimum nature of the diet as highlighted under the weight loss section. Additionally, the similarity between the control and glycine groups may indicate that an aspect other than glycine, such as a different component of collagen, is driving this WBC increase. The WBC increase may lead to an increased immune response to counter the effects of potential infections caused by malnutrition (Li et al., 2007). Similarly, the significant increase in platelet distribution width between the baseline and the glycine supplemented diet, which is an indicator of the size variation in the platelets, may be linked to the slight increase in platelet count in the blood. The immune cell range in the current study is similar to what was found in Bechert et al. (2002) in which the cheetahs were also fed a variety of supplemented meat over a 12 month period, confirming that the cheetahs from the present study were within a normal immune response range.

Glycine is crucial in the biosynthesis of porphyrins in which eight molecules of glycine are required for the biosynthesis of one haem group (ferroporphyrin) (Meléndez-Hevia et al., 2009). In humans, an estimated 240 mg/day glycine is consumed in the production of porphyrins which are mostly used for the haem of haemoglobin or myoglobin, or for the production of catalase and peroxidase (Meléndez-Hevia et al., 2009). In this study, there was no difference in the RBC or related parameters in response to the glycine supplement. As the average RBC, haemoglobin and haematocrit values were in the higher range of what was found in the reference literature, this may indicate that the healthy cheetahs in this study were at a maximum capacity and that the additional glycine was used in the biosynthesis of other metabolites such as purines or creatine.

3.3.3. Serum biochemistry

There was a decrease in the TSP driven by the decrease in albumin from baseline to the control and glycine supplemented diets. Although the meat protein content was the same throughout, prior to the start of the study the cheetahs were fed bones, organs and skin which would provide a more diverse source of amino acids. The potential reduction in the dietary protein source, and therefore amino acids, may have led to a reduction in the serum albumin biosynthesis rate which was observed in rats fed high or low protein diets – with very little change to the catabolism rate until a critical level is reached (Kirsch et al., 1968). In human patients, the relationship between nutritional status, body composition and serum protein and albumin were investigated (Forse and Shizgal, 1980). A highly significant positive correlation was found between body cell mass (which is the total oxygen consuming cells of the body as determined by body mass and body potassium, and is accompanied by the expansion of extracellular mass) and TSP, as well as the serum albumin. The authors concluded that although nutritional status is an important determinant of serum albumin concentration, there are other factors such as malnutrition, malabsorption, liver failure, wounds, and infections that may be contributing factors. Therefore, the decrease in TSP and serum albumin is likely linked to the cheetah's weight loss and the sub-optimum diet with the supplementation of glycine not providing the variety of amino acids required to maintain the serum protein levels.

There was a significant decrease in ALP concentration from baseline to the control and glycine supplemented diet. Although not significant, the same trend was seen with ALT. Both are predominantly liver enzymes that will leak into the bloodstream when there is liver injury or damage (Fernandez and Kidney, 2007). Therefore, a decrease in these enzymes may indicate improved liver functioning or less damage in response to the control and glycine supplemented diet. However, there are various physiological factors that could increase ALP activity — such as age (with juvenile cats and dogs having higher concentrations than adults), pregnancy and lactation and a high fat diet (which has been found in humans), although these factors are not apparent in the current study (Fernandez and Kidney, 2007). Interestingly, the ALP concentration in the study by Bechert et al. (2002) was higher than what was found in the current study. This result might be partly due to older

cheetahs being included in that study or due to the higher fat content of the commercial diets. The generally lower ALP and ALT found in the current study may simply indicate that these cheetahs are indeed young and healthy.

The potassium concentration of the control diet was significantly higher than at baseline and with the glycine supplemented diet. Although all values were in the reference ranges and therefore not indicative of hypo or hyperkalaemia. The total serum calcium concentration was significantly higher at baseline compared to the glycine supplemented diet. This decrease may be directly attributed to the lower serum albumin concentration found in glycine supplemented cheetahs, which would lead to less bound calcium in the serum. Throughout the study, no bones were provided to the cheetahs which would conventionally be a major source of calcium and highlights the importance of calcium supplementation for captive cheetahs fed exclusively meat based diets (Depauw et al., 2012b). The supplement provided to the cheetahs in this study had sufficient levels of calcium and would unlikely contribute to the calcium decrease. Different combinations of immobilisation drugs, such as ketamine-medetomidine or zolazapam-tiletamine-medetomidine, have been shown to cause hypertension and hypoxaemia with the latter combination causing elevated blood calcium, potassium and glucose concentrations (Buck et al., 2022). This drug combination was used in the current study and may have attributed to the differences found in the serum biochemistry parameters, but is highly unlikely, as the same drug combinations were used during all three immobilisation events.

CHAPTER FOUR

4. URINE METABOLITES

4.1. Introduction

Urine, in mammals, is a transparent and sterile fluid generated by the kidneys and consists of a variety of compounds at a high concentration (Bouatra et al., 2013). These compounds include urea, inorganic salts, creatinine, ammonia, organic acids, various water soluble toxins and pigmented products of haemoglobin. The medical diagnostic value of urine has been explored since 4000 BC with a total of 2651 metabolites identified and quantified in the urine of humans (Bouatra et al., 2013). In metabolomics studies, urine is the most common sample used due to its metabolite abundance, easy non-invasive collection, limited sample preparation and limited sample pre-treatment owing to its lower complexity and protein content (Zhang et al., 2012). Urinary metabolites, which are end products of normal and pathological cellular processes in humans and animals, are closely linked to phenotypes and act as an ideal proxy for the metabolic activity of the organs (i.e. kidney) from which it is derived (Wishart, 2019; Zhang et al., 2012). Urine metabolomics has successfully been used in a range of fields, such as physiology, diagnostics, functional genomics, pharmacology, toxicology and nutrition (Zhang et al., 2012). Importantly, urine metabolomics is emerging as a significant tool in the discovery of biomarkers and the diagnosis of many diseases including jaundice syndrome, various cancers, chronic obstructive pulmonary disease (COPD), renal injury and diabetes (Zhang et al., 2012).

There have been a limited number of studies investigating the urine metabolites of captive or free-ranging cheetahs. Burger et al. (2006) utilised GC-MS to investigate the volatile constituents in the urine headspace vapour of captive male and female cheetahs and found that elemental sulphur (S_6 and S_8) was present in all samples. The authors concluded that this may be another mechanism of sulphur excretion or that it allows cheetahs to avoid detection from prey and predators due to its odourless secretion. The study by Tordiffe et al. (2017) detected 339 organic acids in the urine of healthy captive cheetahs, of which, phenolic compounds linked to the anaerobic fermentation of aromatic amino acids in the intestine and glycine conjugates were present in high concentration. Furthermore, Tordiffe and Mienie (2019) identified 38 amino acids in the urine of captive cheetahs with arginine having nearly twice the concentration of the next amino acid, glutamine. Arginine is an essential amino acid which cats are unable to produce endogenously and, therefore, require substantial amounts from the diet in order to maintain the ornithine reserves for ammonia detoxification in the urea cycle. Cystine, which is reduced in urine to form two cysteine molecules, had the highest fractional excretion (22%) which indicates that a high percentage of cystine is excreted and not reabsorbed by the body. This may be due to exogenous methionine and serine

from the consumed animal tissue meeting the high metabolic demand of glutathione, taurine, CoA and general protein synthesis.

Glycine has many physiological functions and, as a result, there are many glycine linked metabolites that may be found in urine, most notably those associated with glycine conjugation (Badenhorst et al., 2014). Hippurate (or hippuric acid) is the most abundant amino acid conjugate found in the urine of mammals and is formed with the conjugation of glycine and benzoate. Although benzoate, salicylate, 4-hydroxybenzoate, 3-hydroxybenzoate, 4-aminobenzoate and 2-furoate are the natural substrates for conjugation, the major source of glycine conjugation substrates seems to be metabolites of dietary polyphenols produced by microorganisms in the gut (Badenhorst et al., 2014). These compounds include phenylpropionate, 3-hydroxyphenylpropionate and 4-hydroxyphenylpropionate. The amount of glycine conjugates excreted in urine depends on the dietary intake of polyphenolic compounds and their extent of fermentation in the colon (Badenhorst et al., 2014). Glycine forms part of the biosynthesis of carnitine and creatine which can also be excreted in urine, the latter as creatinine (Meléndez-Hevia et al., 2009). Glutathione and benzoate metabolism are indicators of glycine deficiency, with increased excretion of pyroglutamic acid (5-oxoproline) and hippurate acting as markers, respectively (Meléndez-Hevia et al., 2009).

Considering the limited urinary metabolomics studies conducted in cheetahs and the potential benefits of using this approach to investigate the metabolic effect of glycine, the aims of this chapter are to:

- identify and quantify (through relative concentrations) the metabolites present in the urine of 10 captive cheetahs at baseline, on a control diet and on a glycine supplemented diet;
- highlight and discuss the identified metabolites of importance.

4.2. Results

4.2.1. Identified metabolites and relative concentration

ASICS identified a total of 151 metabolites across all 30 urine samples. After data cleaning, 25% of the metabolites were removed and 112 metabolites remained. The 30 most abundant metabolites for each group and their relative concentrations are indicated in Table 6 and all identified metabolites are shown in Supplementary Table S2. The most abundant metabolite across all three groups was creatinine with a five to six times higher relative concentration than allantoin, the next abundant metabolite. L-cysteine and malonic acid were also abundant across all three groups, while L-ornithine (baseline), TMAO (control) and glycolic acid (glycine diet) were abundant in the respective diets. As displayed in Table 6, glycolic acid and guanidinoacetic acid were abundant in the glycine supplemented diet in 7 individuals, but interestingly, was present in less than 5

individuals in the baseline and control diet. It was, therefore, omitted from the baseline and control groups in Table 6.

Table 6. The 30 most abundant metabolites identified by NMR in the urine of 10 captive cheetahs at baseline, on a control diet and on a glycine supplemented diet.

Baseline			Control diet			Glycine supplemented diet		
Metabolite	**n**	**Relative concentration**	**Metabolite**	**n**	**Relative concentration**	**Metabolite**	**n**	**Relative concentration**
Creatinine	10	64.1 ± 47.4	Creatinine	10	72.8 ± 37.6	Creatinine	10	56.9 ± 46.2
Allantoin	10	14.3 ± 3.2	Allantoin	10	13.7 ± 2.9	Allantoin	10	15.2 ± 3.8
L-Cysteine	10	10.5 ± 0.7	Malonic acid	10	13.2 ± 4.9	L-Cysteine	10	10.1 ± 1.6
Malonic acid	10	7.8 ± 4.7	TMAO	10	10.2 ± 7.2	Glycolic acid*	7	9.6 ± 6.2
L-Ornithine	10	6.1 ± 1.3	L-Cysteine	10	9.9 ± 0.9	Malonic acid	10	9.6 ± 4.0
L-Proline	10	4.5 ± 0.4	L-Carnitine	10	7.3 ± 2.6	TMAO	10	7.3 ± 4.7
DHA	10	4.3 ± 1.1	L-Ornithine	10	5.8 ± 0.9	Guanidinoacetic acid*	7	6.8 ± 5.2
L-Carnitine	10	3.9 ± 2.3	L-Proline	10	5.5 ± 0.7	L-Ornithine	10	6.4 ± 1.2
TMAO	10	3.9 ± 3.6	Taurine	10	4.4 ± 1.4	L-Proline	10	5.1 ± 0.5
1,3-DAP	10	3.4 ± 0.3	DHA	9	3.9 ± 0.9	L-Carnitine	10	4.9 ± 2.2
L-Tryptophan	8	3.1 ± 0.6	Choline chloride	10	3.8 ± 1.2	DHA	10	4.1 ± 0.8
L-Arginine	10	3.1 ± 0.5	Phosphocholine	10	3.5 ± 0.9	Taurine	10	3.8 ± 1.1
Taurine	10	3.0 ± 1.1	α-Ketoglutaric acid	10	3.4 ± 0.4	L-Glutamine	10	3.2 ± 0.8
Choline chloride	10	3.0 ± 1.1	L-Glutamine	10	3.4 ± 1.1	Choline chloride	10	3.2 ± 1.1
α-Ketoglutaric acid	10	2.7 ± 0.4	D-Glucose	10	3.2 ± 0.6	1,3-DAP	10	3.1 ± 0.3
D-Maltose	10	2.6 ± 0.4	D-Maltose	10	3.2 ± 0.5	L-Arginine	10	3.0 ± 0.3
D-Glucose	10	2.6 ± 0.4	L-Arginine	10	3.2 ± 0.7	α-Ketoglutaric acid	10	2.9 ± 0.3
L-Lysine	9	2.6 ± 0.4	Dimethylglycine	10	3.2 ± 2.1	L-Tryptophan	10	2.8 ± 0.6
Phosphocholine	10	2.6 ± 1.0	Creatine	7	3.1 ± 2.8	Phosphocholine	10	2.8 ± 0.7
L-Citrulline	10	2.6 ± 0.2	1,3-DAP	10	2.9 ± 0.3	D-Maltose	10	2.6 ± 0.6
L-Glutamine	10	2.5 ± 0.9	Threonic acid	8	2.7 ± 1.0	Threonic acid	9	2.6 ± 0.7
D-Galactose	9	2.5 ± 1.1	L-Tryptophan	9	2.7 ± 0.6	L-Lysine	8	2.5 ± 0.4
β-Alanine	10	2.4 ± 0.3	β-Alanine	10	2.7 ± 0.2	D-Glucose	10	2.5 ± 0.6

D-Gluconic acid	8	2.4 ± 0.8	D-Gluconic acid	7	2.6 ± 0.9	β-Alanine	10	2.5 ± 0.3
Threonic acid	9	2.0 ± 0.7	1-Methyl-hydantoin	10	2.6 ± 2.3	L-Citrulline	10	2.5 ± 0.3
Glycero-phosphocholine	10	1.9 ± 0.6	L-Citrulline	10	2.6 ± 0.4	Glycero-phosphocholine	10	2.1 ± 0.5
Phenethylamine	10	1.8 ± 0.5	L-Asparagine	9	2.6 ± 1.1	Phenethylamine	10	1.9 ± 0.3
Glycogen	10	1.8 ± 0.2	L-Lysine	8	2.5 ± 0.4	Glycogen	10	1.8 ± 0.3
Lactose	10	1.7 ± 0.5	Glycero-phosphocholine	10	2.4 ± 0.5	Lactose	10	1.8 ± 0.8
L-Cystine	10	1.6 ± 0.3	Phenethylamine	10	2.2 ± 0.9	L-Cystine	10	1.7 ± 0.3

*Data are expressed as mean ± SD. n indicates the number of samples where the metabolite was detected. Only those present in >5 samples were included in the table. * indicates metabolites that were present at high relative concentration in <5 samples of the baseline and control diets. Relative concentration is an arbitrary unit. TMAO, Trimethylamine oxide; 1,3-DAP, 1,3-Diaminopropane; DHA, dehydroascorbic acid*

4.2.2. Metabolites identified by univariate analysis

MetaboAnalyst identified 31 significant metabolites with a one-way repeated measures ANOVA. The significant metabolites as well as their post hoc analysis conducted in JMP are shown in Table 7. Importantly, these metabolites are not necessarily those that were the most abundant in Table 6. Dimethylamine had the lowest adjusted p-value ($p<0.001$) with the control and glycine supplemented diet significantly greater than at baseline. The relative concentrations of the control diet were greater than baseline for 22 of the identified metabolites, with only 1,3-diaminopropane having a higher concentration at baseline compared to the control diet. Similarly, the relative concentrations of the control diet were higher than the glycine supplemented diet for 11 metabolites with the glycine diet being greater than the control for fructose and CMP. For most of the metabolites, the baseline and glycine supplemented diets were the same with the exceptions being dimethylamine (higher in the glycine diet) and methylmalonic acid (higher at baseline). While L-cystine, isovaleric acid, phosphocholine, pimelic acid and hypotaurine had an overall significance identified with ANOVA, but this was not shown in the post hoc analysis between groups presumably due to the different statistical programmes.

Table 7. Significant metabolites identified by univariate analyses in the urine of captive cheetahs at baseline, on a control diet and glycine supplemented diet (n=10)

Metabolite	**F-value**	**Adjusted p-value**	**Tukey-Kramer p-value**		
			Baseline-Control	**Baseline-Glycine**	**Control-Glycine**
Dimethylamine	28.56	0.0003	**<0.01** (C>B)	**0.01** (G>B)	NS
α-Ketoglutaric acid	19.64	0.0016	**<0.01** (C>B)	NS	**0.02** (C>G)
Methylguanidine	15.91	0.0037	**< 0.01** (C>B)	NS	**<0.01** (C>G)
L-Carnitine	14.86	0.0037	**0.01** (C>B)	NS	NS
Dimethyl sulphone	14.53	0.0037	**<0.01** (C>B)	NS	**<0.01** (C>G)
L-Proline	12.57	0.0064	**<0.01** (C>B)	NS	NS
L-Cystine	12.35	0.0064	0.05	NS	NS
Pyroglutamic acid	11.95	0.0066	**<0.01** (C>B)	NS	NS
Trimethylamine	10.96	0.0084	**<0.01** (C>B)	NS	**<0.01** (C>G)
Methylmalonic acid	10.89	0.0084	NS	**0.04** (B>G)	NS
L-Asparagine	10.30	0.0100	**0.02** (C>B)	NS	**0.01** (C>G)
Trans-Aconitic acid	9.71	0.0122	**0.02** (C>B)	NS	NS
L-Carnosine	9.51	0.0124	**<0.01** (C>B)	NS	NS
D-Fructose	9.31	0.0125	NS	NS	**<0.01** (G>C)
D-Maltose	9.19	0.0125	**0.03** (C>B)	NS	**0.02** (C>G)
Taurine	8.32	0.0183	**0.04** (C>B)	NS	NS
Isovaleric acid	7.41	0.0280	NS	NS	NS
Malonic acid	7.25	0.0288	**0.04** (C>B)	NS	NS
β-Alanine	6.94	0.0324	**0.03** (C>B)	NS	NS
Acetoacetic acid	6.84	0.0327	**0.01** (C>B)	NS	NS
L-Valine	6.53	0.0357	**0.04** (C>B)	NS	**0.01** (C>G)
3-Hydroxyphenylacetic acid	6.52	0.0357	NS	NS	**0.04** (C>G)
Phosphocholine	6.41	0.0365	NS	NS	NS
Dimethylglycine	6.17	0.0396	**<0.01** (C>B)	NS	**<0.01** (C>G)
Pimelic acid	6.13	0.0396	NS	NS	NS
D-Glucose	5.72	0.0483	**0.03** (C>B)	NS	**0.02** (C>G)
1-Methylhydantoin	5.67	0.0483	**<0.01** (C>B)	NS	**0.02** (C>G)
Hypotaurine	5.57	0.0496	NS	NS	NS
CMP	5.49	0.0496	NS	NS	**0.04** (G>C)
TMAO	5.46	0.0496	**0.04** (C>B)	NS	NS
1,3-DAP	5.40	0.0499	**0.04** (B>C)	NS	NS

NS, not significant; CMP, cytidine monophosphate; *TMAO, Trimethylamine oxide; 1,3-DAP, 1,3-Diaminopropane*

4.2.3. Metabolites identified by multivariate analysis

The PCA and PLS-DA score plots are shown in Figure 4. Principal component (PC) 1 accounts for 27.5% of the variation seen across the data, while PC 2 accounts for 9.5% of the variation. The PCA plot shows overlap with all three diets, with the control diet having a greater spread. Based on the spread of the data, the metabolites driving the variation in PC 1 (i.e. the spread on the x axis) are contributing more to the baseline and glycine diet, while the metabolites driving the variation in PC 2 (i.e. the spread on the y axis) are contributing similarly to PC 1 for the control diet. All the metabolites would contribute to the variation, however the loadings or contribution of the top 10 metabolites of PC 1 and PC 2 are shown in Table 8. Pantothenic acid, L-carnitine, malonic acid and choline chloride were the metabolites with the highest loading scores for PC 1, therefore contributing the most to the axis distribution. In contrast, UDPG, glutaconic acid, trimethylamine and D-fructose had the highest loadings for PC 2.

The separation seen on the PLS-DA plot between the diets is noticeably small, with the control diet overlapping with both the baseline and glycine diet. This is confirmed with the negative Q2 values (Supplementary Figure S1) which indicate that this model is not appropriately predicted or is overfitted for each diet. As with the PCA plot, the control diet has a wide spread of data. The discriminant variables of component 1 accounts for 18.4% of the variation between the diets, while component 2 accounts for 15.1% of the variation seen between diets. The difference between the glycine supplemented and baseline diets account for the greatest separation along component 1 and component 2, respectively. The overall VIP scores and the coefficient variables of each diet for the first 10 metabolites are shown in Table 8. Dihydrothymine, methylmalonic acid, dimethylamine and pyroglutamic acid were the variables with the greatest importance in both component 1 and 2 in the PLS-DA model. Due to the discriminatory nature of PLS-DA in separating according to groups, the coefficients of regression indicate the contribution of each metabolite to the predicted model for each diet. Dihydrothymine, dimethyl sulphone and dimethylamine had the highest weighting at baseline. Methylguanidine, dimethyl sulphone and trimethylamine contributed to the control diet. Similarly to the baseline diet, dihydrothymine had a large contribution towards the glycine supplemented diet. This was followed by dimethyl sulphone and N-(2-Furoyl)Glycine. A more extensive list for the multivariate analyses is provided in Supplementary Table S3.

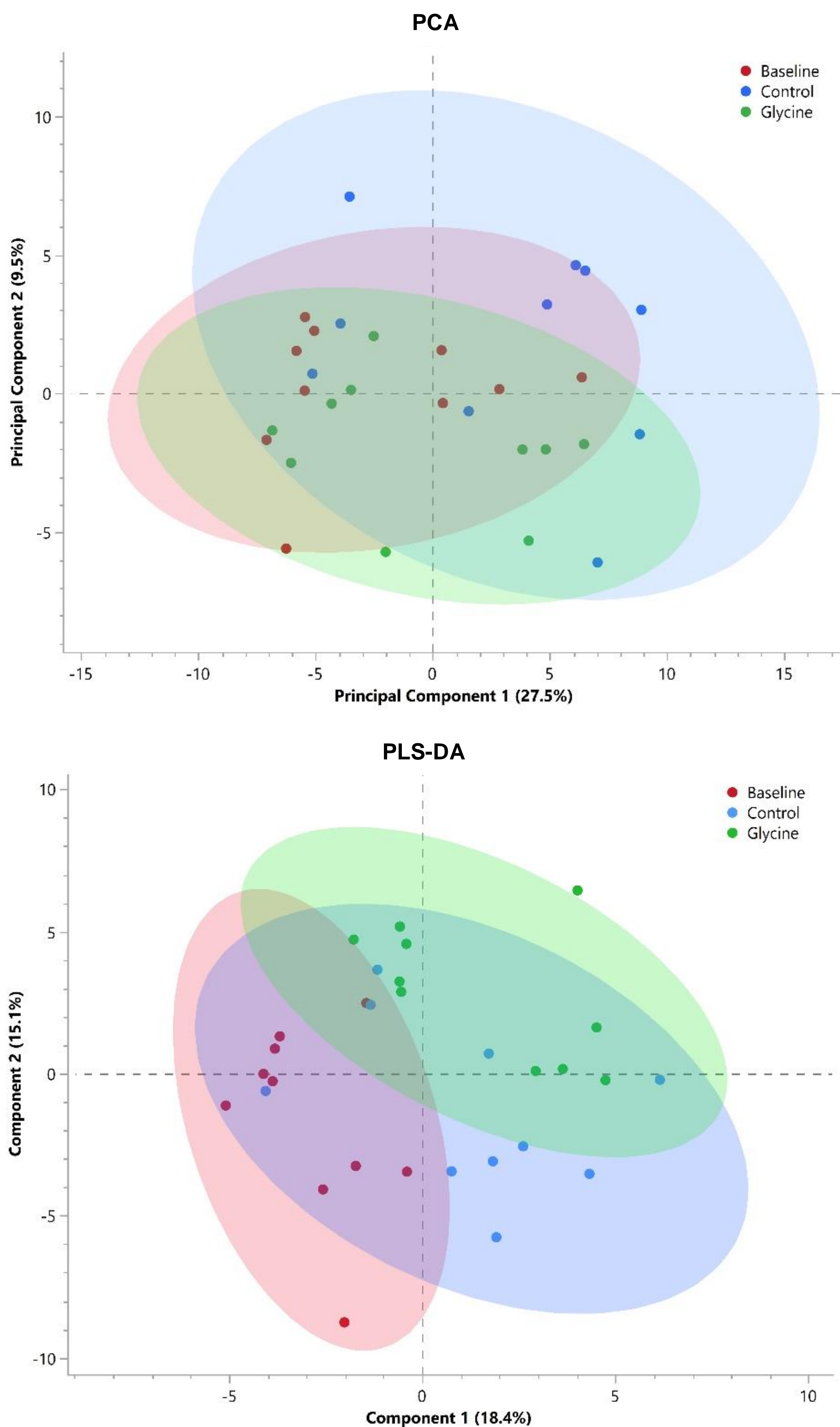

Figure 4. A two dimensional PCA (top) and PLS-DA (bottom) scores plot of the metabolites identified in the urine of 10 captive cheetahs at baseline, on the control and glycine supplemented diets. *Each point represents an individual cheetah at baseline (red), control (blue) and glycine supplemented (green) with a corresponding 95% confidence ellipse for each diet.*

Table 8. The (A) PCA loadings, (B) PLS-DA VIP scores and (C) PLS-DA diet regression coefficients for the top 10 urinary metabolites in 10 captive cheetahs.

A. PCA Metabolite loadings				B. PLS-DA Metabolite VIP Scores			
PC 1		PC 2		C 1		C 2	
Pantothenic acid	-0.17	UDPG	-0.22	Dihydrothymine	2.63	Dihydrothymine	2.19
L-Carnitine	0.17	Glutaconic acid	-0.21	Methylmalonic acid	2.40	Methylmalonic acid	1.79
Malonic acid	0.17	Trimethylamine	0.20	Dimethylamine	2.36	Dimethylamine	1.75
Choline chloride	0.16	D-Fructose	-0.19	Pyroglutamic acid	2.13	Pyroglutamic acid	1.65
Acetoacetic acid	0.16	Spermidine	0.19	L-Proline	2.02	GMP	1.59
L-Proline	0.16	Dimethylglycine	0.19	GMP	1.94	N-(2-Furoyl)Glycine	1.57
Isovaleric acid	-0.16	Methylguanidine	0.18	1,3-DAP	1.93	L-Proline	1.55
Acetaminophen	0.15	Lactic acid	-0.18	D-Fucose	1.89	Adipic acid	1.52
Taurine	0.15	L-Histidine	-0.18	Dimethyl sulphone	1.69	1,3-DAP	1.47
Ethanolamine	0.15	2-Deoxycytidine	0.17	L-Glutamine	1.61	UMP	1.45

C. PLS-DA metabolite coefficients for each diet					
Baseline		Control		Glycine	
Dihydrothymine	97.8	Methylguanidine	71.9	Dihydrothymine	100.0
Dimethyl sulphone	88.1	Dimethyl sulphone	68.1	Dimethyl sulphone	79.8
Dimethylamine	84.4	Trimethylamine	60.9	N-(2-Furoyl)Glycine	75.8
N-(2-Furoyl)Glycine	71.7	Dimethylglycine	56.2	GMP	74.1
1,3-DAP	71.7	α-Ketoglutaric acid	52.1	Pimelic acid	68.3
Methylmalonic acid	71.2	D-Fructose	50.1	Dimethylamine	68.0
GMP	70.9	1-Methylhydantoin	49.1	Methylmalonic acid	64.8
Pimelic acid	63.7	D-Glucose	46.9	1,3-DAP	60.6
L-Proline	61.5	Dimethylamine	46.8	Adipic acid	59.4
UMP	60.4	L-Valine	44.6	L-Tryptophane	54.8

Loadings are the weight of each metabolite and are expressed as positive or negative number depending on its direction from the PC. Coefficients are the weighted sum of the PLS-DA regression for each group. PCA, principle component analysis; PLS-DA, partial least squares discriminant analysis, VIP, variable importance in projection; PC, principle component; C, component; TMAO, trimethylamine oxide; UDPG, uridine 5'-diphosphate glucose; GMP, guanosine monophosphate; UMP, uridine monophosphate; CMP, cytidine monophosphate; 1,3-DAP, 1,3-diaminopropane

4.2.4. Summary of potentially important metabolites

After the univariate and multivariate approach, the initial 112 urinary metabolites were reduced to 65 metabolites (Supplementary Table S4). These metabolites were present in the top 20% of at least one of the analyses. Ten metabolites were identified in 5 or more analyses – dimethyl sulphone, L-proline, D-fructose, dimethylamine, trimethylamine, pyroglutamic acid, 1,3-diaminopropane, dihydrothymine, methylmalonic acid and pimelic acid. The normalised concentration of the 10 identified metabolites at baseline, on the control and glycine supplemented diet are shown in Figure 5. Methylmalonic acid and 1,3-diaminopropane had significantly higher

concentrations at baseline compared to the glycine and control diets, respectively. L-proline and pyroglutamic acid had significantly higher concentrations on the control diet compared to baseline, while fructose had a higher concentration on the glycine diet compared to the control diet. Dimethyl sulphone and trimethylamine had higher concentrations on the control diet compared to both the baseline and glycine supplemented diets. Dimethylamine had a higher concentration on the control and glycine supplemented diet compared to the baseline diet. Significance was not identified univariately between groups for dihydrothymine and pimelic acid. Interestingly, dihydrothymine was identified in 9 cheetahs on the glycine diet, but only 5 and 6 individuals on the baseline and control diets. Similarly, fructose was identified in 10 cheetahs on the glycine diet, but in only 8 and 3 cheetahs on the baseline and control diets, respectively.

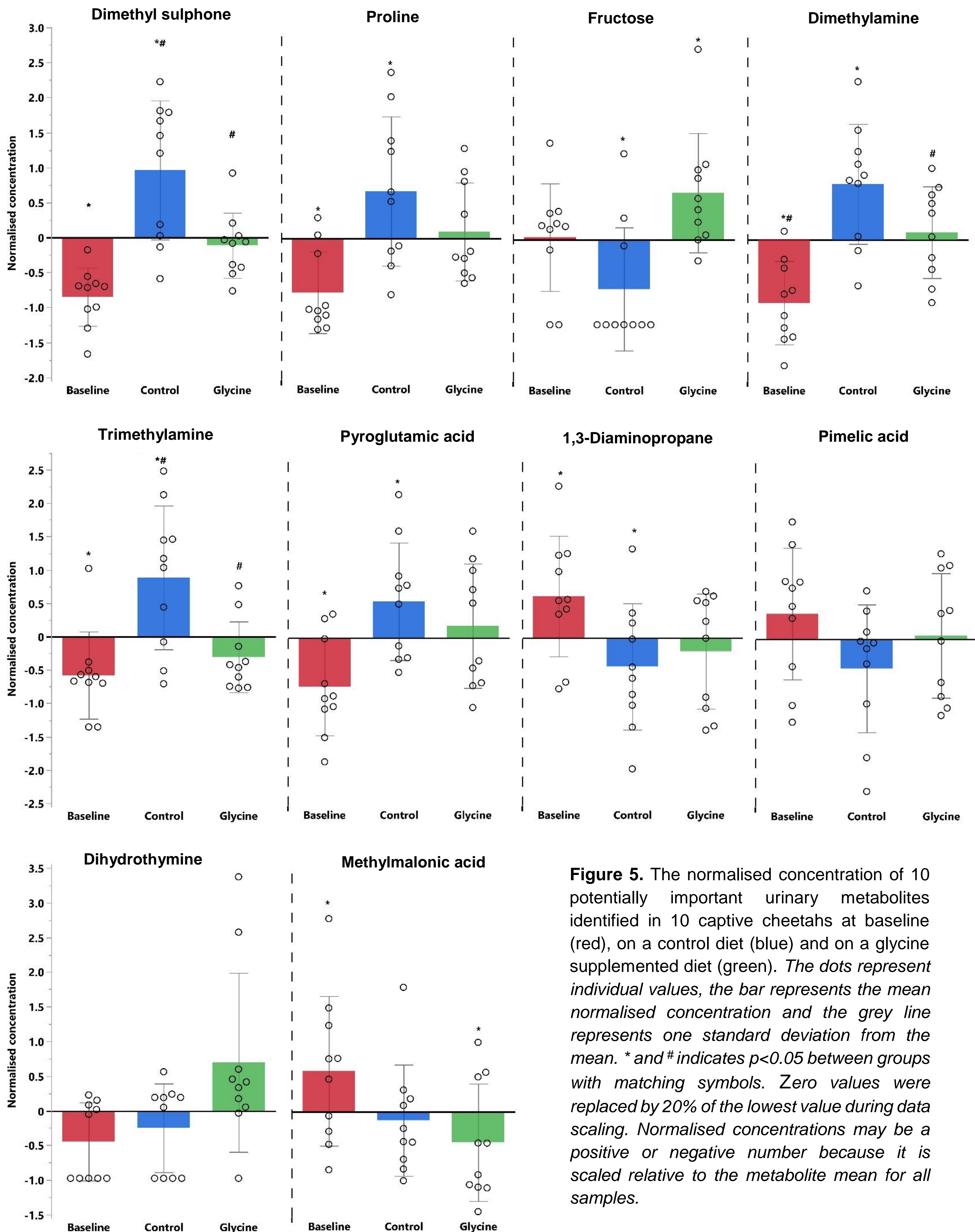

Figure 5. The normalised concentration of 10 potentially important urinary metabolites identified in 10 captive cheetahs at baseline (red), on a control diet (blue) and on a glycine supplemented diet (green). *The dots represent individual values, the bar represents the mean normalised concentration and the grey line represents one standard deviation from the mean. * and # indicates p<0.05 between groups with matching symbols. Zero values were replaced by 20% of the lowest value during data scaling. Normalised concentrations may be a positive or negative number because it is scaled relative to the metabolite mean for all samples.*

4.3. Discussion

4.3.1. Overview of identified metabolites in urine

In the current study, a total of 151 metabolites were identified in the urine of captive cheetahs of which 39 metabolites were removed because they were present in less than 50% of the samples in each group. Due to the untargeted approach, these metabolites span multiple classes of compounds, such as amino acids, organic acids and compounds, fatty acids, acylcarnitines and sugars. The identification of glycogen and acetaminophen as listed in Table 6 and 8 were somewhat surprising as the former is a large polysaccharide and the latter is paracetamol that is often administered as a drug. Cheetahs have been found to excrete relatively large molecules, which may include polysaccharides like glycogen although this hasn't been found previously (Tordiffe et al., 2017). Alternatively, this may be a misidentification of another glucose derivative. Similarly, paracetamol can occur naturally in plants which may be ingested by cheetahs in their enclosures, but whether this would present in their urine is not apparent. None of these metabolites were highlighted in multiple analyses. The identification of 112 metabolites in the current study aligns with previous human studies in which an average of 100 urine metabolites are identified per study across different platforms (Bouatra et al., 2013). Additionally, sex was not specifically investigated in the present study due to the randomised cross-over design which allowed for matched comparison between groups and reduced any confounding effects caused by sex. Tordiffe et al. (2016, 2017, 2019) found no significant difference in urinary amino acids, serum amino acids and serum fatty acids between male and female captive cheetahs. For urinary organic acids, 6 organic acids out of a potential 199 were highlighted as contributing significantly towards the separation of males and females using PCA and PLS-DA – namely N-acetylphenylalanine, 2-hydroxy-5-methoxybenzoic acid, N-hexanoylglycine, N-tiglylglycine, 2,3-dihydroxybenzoic acid and furoylglycine (Tordiffe et al., 2017). Interestingly, these are all associated with glycine or benzoic acid, but they were not identified in other analyses and, therefore, may simply be indicative of the high abundance of glycine conjugates found in the study. Overall, these three studies found that captivity and age had a greater association with the identified metabolites and that the influence of sex was minor.

Creatinine is commonly used to control for the effects of urine volume on total urine concentration because, in the absence of kidney disease, it is believed to be excreted at relatively constant and unchanged amounts from the kidneys (Ryan et al., 2011). However, there are challenges with this approach, primarily regarding the day-to-day variability in excretion due to exogenous factors such as diet, sex, age, time of day and disease. Secondly, considering the large number of metabolites identified in metabolomics studies at varying concentrations, it may not be suitable to normalise an entire metabolome to a single metabolite, i.e. a more holistic approach should be taken (Ryan et al., 2011). In healthy captive cheetahs, the urine creatinine concentration was highly variable and largely influenced by age with a peak reached between four and six years old (Tordiffe et al., 2017).

Tordiffe et al. (2017) concluded that normalising urine metabolite concentrations to creatinine would potentially lead to the overestimation of metabolite concentrations in older and younger cheetahs, and suggested the use of urine specific gravity where the variability was lower due to the tight regulation of water in carnivorous mammals. Since the creatinine variability in the current study was quite low (186 ± 25 µmol/L) across all three diets and the urine specific gravity fairly constant (1069 ± 6 au) urine metabolites were not normalised to these values. Instead, the urine data was normalised during the pre-processing steps using the PQN method which adjusts the individual metabolites in relation to the NMR spectrum. This procedure was performed after the removal of the unwanted water and urea regions in the spectrum (Dieterle et al., 2006).

There were a total of 65 urine metabolites identified across multiple analyses of which 31 were significant in univariate analysis. The sections below will focus on the 10 identified metabolites of interest, which were highlighted in five or more analyses, and discuss their potential link to glycine supplementation.

4.3.2. Dimethyl sulphone ($DMSO_2$)

$DMSO_2$ (also known as methylsulfonylmethane, MSM) was present in the urine of all 10 cheetahs at baseline, on the control diet and on the glycine supplemented diet, and was identified as important across both univariate and multivariate analyses. $DMSO_2$ is a natural organic sulphur compound that was identified in normal human urine in 1966 and has since been identified in the urine, blood, cerebrospinal fluid, skin, sweat and earwax of various species including cows, rats, bobcats, African wild dogs, red deer and cheetahs (Burger et al., 2006; He and Slupsky, 2014). The concentrations of $DMSO_2$ in the biofluids of humans are highly influenced by diet with the consumption of foods such as onions, grains and fibres, cabbage, meats, eggs, dairy and fish leading to increased concentrations of $DMSO_2$ (He and Slupsky, 2014; Trimigno et al., 2020). In a comprehensive urinary NMR metabolomics study conducted in a Danish population, $DMSO_2$ was a key discriminatory metabolite of the conventional Danish diet characterised by sugary products, refined grains (pasta and rice included), meat, dairy, and low-fibre vegetables (Trimigno et al., 2020). The authors concluded that the notable presence of $DMSO_2$ was likely due to the contrast in gut microbial metabolism between the conventional Danish diet and the New Nordic diet rich in fish, whole grains and organic fruit and vegetables. Additionally, in a recent study investigating the serum metabolome of patients with chronic kidney disease–mineral and bone disorder it was found that increased $DMSO_2$ was the most notable metabolite in patients with high bone turnover (Baptista et al., 2020). The authors hypothesised that this may be due to its role in osteogenesis which was found in earlier *in vitro* studies, but the direct link to bone turnover and mineralisation is not clear. Interestingly, glycine was also increased in patients with high bone turnover and is likely due to increased collagen turnover (Baptista et al., 2020).

The origin of $DMSO_2$ is believed to be microbial and, while the pathways are poorly understood, evidence suggests that $DMSO_2$ production is linked to the decomposition of methionine by microbiota in the gastrointestinal tract (He and Slupsky, 2014). Dietary methionine that escapes absorption in the small intestine is then subject to absorption and catabolism by colonic bacteria which converts it to the highly reactive methanethiol. Methanethiol is either non-enzymatically converted to dimethyl disulphide or dimethyl trisulfide, or enzymatically (via the host enzymes) converted to dimethyl sulphide. The latter pathway ultimately leads to the production of $DMSO_2$ which is one of the major end products of the methionine degradation pathway (He and Slupsky, 2014). In patients with inflammatory bowel disease, $DMSO_2$ was earmarked as an important biomarker as it was significantly lower in the serum of these patients compared to healthy controls (Dawiskiba et al., 2014). This potentially indicates the role of the microbial methionine degradation pathways in intestinal disease. Glycine was also highlighted as a key biomarker as it was increased in inflammatory bowel disease patients.

$DMSO_2$ has previously been identified in the urine headspace vapour of five captive cheetahs using GC-MS (Burger et al., 2006). The authors hypothesised that the excretion of organic sulphur compounds such as $DMSO_2$, which is an odourless compound, is linked to territory marking and allows cheetahs to be undetected in close proximity to prey and predators. However, this study did not investigate the role of diet and disease in this small sample. In the current study, $DMSO_2$ was significantly higher in the urine of the control diet compared to both the baseline and glycine supplemented diets. The amount of methionine received by the cheetahs would have remained constant throughout the study and other potential sources of related organic sulphur compounds would be negligible (Table 1). Therefore, the increased excretion of $DMSO_2$ in the control diet is likely linked to the increased catabolism of methionine by the gut microbiota. This effect is potentially corrected when cheetahs undergo glycine supplementation leading to reduced production of $DMSO_2$ as the exogenous source of glycine reduces the pressure on endogenous glycine biosynthesis, in which methionine is an intermediate required for the biosynthesis of glycine from choline (Wang et al., 2013). The baseline diet represents a more optimum metabolite picture because the cheetahs received organ mince, skin and bones three weeks prior to the sampling. Therefore, the lower $DMSO_2$ reflects the remnants of an optimal diet in captivity, much like lower urine concentrations reflected the healthier Nordic diet in a Danish population (Trimigno et al., 2020). In the current study, $DMSO_2$ was the most prevalent metabolite identified across multiple analyses which highlights its importance in this captive cheetah population. This is particularly important when the high prevalence of gastrointestinal disease in captive cheetahs is considered and its potential link to diet (Lane et al., 2012; Munson et al., 1999).

4.3.3. L-Proline

Proline was identified in the urine of all 10 cheetahs across the baseline, control and glycine supplemented diet and was significantly higher in the control diet compared to baseline. The individuals on the glycine diet had a similar normalised concentration distribution to those on the control diet (Figure 5). Proline is a rigid amino acid with a unique cyclic structure which allows it to play a vital structural role in collagen along with other metabolic and physiological roles such as an antioxidant and signalling molecule (Li and Wu, 2018). Along with glycine and hydroxyproline (which is a metabolite of proline), proline plays a direct role in the biosynthesis of collagen which is the most abundant protein in mammals. Proline can conventionally be endogenously synthesised from arginine (from ornithine), glutamate and glutamine, but the lack of pyrroline-5-carboxylate synthase in cats does not allow proline production from glutamate and glutamine (Wu et al., 2011). Therefore, dietary proline is essential for growth and health maintenance. Dietary protein bound proline and hydroxyproline are hydrolysed by specific proline peptidases in the gastrointestinal tract of the small intestine to yield free proline or hydroxyproline (Wu et al., 2011). Roughly 40% of the luminal proline is catabolised by enterocytes and bacteria with the remaining amount entering portal circulation.

Proline has previously been identified in the serum (108 to 760 µmol/L) and urine (11 to 219 µmol/L) of captive cheetahs using GC-MS (Tordiffe and Mienie, 2019). In urine, the fractional excretion rates of proline-hydroxyproline (5.5%) and glycine-proline (1.7%) were high compared to many other amino acids, while urinary glycine and proline-hydroxyproline concentrations also significantly decreased with age. The authors concluded that since the excretion of free and bound hydroxyproline reflect the breakdown of mature collagen, along with the high concentration of glycine and proline, this may indicate subclinical nutritional deficiencies in young growing cheetahs. In the current study, hydroxyproline (identified as trans-4-Hydroxy-L-proline) was found in the urine across all three diets, but was only identified as an important contributor to variation in PC 2 which would potentially be contributing to the control diet. The higher urinary excretion of proline in the control group may be linked to the sub-optimum diet and collagen breakdown because arginine and ornithine (from which proline can be synthesised) was found at similar concentrations across all three diets.

4.3.4. D-Fructose

Fructose, a simple sugar, was found in the urine of 8 and 10 cheetahs at baseline and on the glycine supplemented diet, respectively. However, it was only identified in three cheetahs on the control diet, which likely contributed to the significant difference found between the control and glycine supplemented diet. The significance of fructose is somewhat surprising considering the carnivorous nature and high protein diet of cheetahs. Fructose can be endogenously synthesised from glucose and sorbitol by the polyol pathway in mammals with its production and metabolism suggested in the pathogenesis of metabolic syndrome and renal disease (Andres-Hernando et al., 2019).

Domestic cats express a hepatic fructokinase enzyme, which metabolises dietary fructose at a higher level than canine liver (Schermerhorn, 2013). This high expression is unexpected considering the low quantity of fructose in the strict carnivore diets of cats, but does align with their ability to digest and absorb fructose in the intestine (Schermerhorn, 2013). In cheetahs, fructose and glucose are important fuel substrates for spermatozoa and are metabolised for energy (Weiner et al., 2019). While fructose has not been identified in relation to cheetahs in any other study, the use of fructose in spermatozoa highlights the potential role of this sugar in reproduction, which may indicate an overall metabolic significance. Cheetahs, like domestic cats, are reliant on the breakdown of gluconeogenic amino acids (such as glycine) and the movement of these products into gluconeogenic pathways to produce energy (Schermerhorn, 2013). Urinary fructose was higher in the glycine supplemented diet compared to the control diet, while glucose was higher in the control group compared to the glycine and baseline group. Glycine supplementation has been shown to play a role in glucose homeostasis by reducing glucose production, increasing glucose tolerance and improving insulin sensitivity (Imenshahidi and Hossenzadeh, 2022). This would explain why the glucose concentration was lower in the glycine and baseline diet, however the inverse link to fructose concentration is not apparent.

4.3.5. Dimethylamine (DMA) and Trimethylamine (TMA)

The secondary and tertiary amine organic compounds, namely DMA and TMA, were identified at low concentrations in the urine of all cheetahs at baseline (excluding two cheetahs for TMA), on the control and glycine supplemented diets. TMA can be synthesised from dietary choline, trimethylglycine, phosphatidylcholine and carnitine, and either oxidised to TMAO or degraded to DMA and formaldehyde by gut microbial enzymes (Chhibber-Goel et al., 2016). DMA can also be synthesised by the post translational modification of arginine in certain proteins which ultimately produces DMA amongst other compounds (Tsikas, 2020).

In the current study, the DMA concentration was significantly lower at baseline compared to both the control and glycine supplemented diets, which aligns with its noticeable contribution to the variation in the baseline group in the PLS-DA model. For TMA, the concentration was significantly higher on the control diet compared to both the baseline and the glycine diet. The concentration pattern of TMA matches DMA which is expected considering that TMA is a major precursor for DMA. Fish and seafood are an important exogenous source of DMA and TMA through choline, although in the cheetah diet the exogenous source would likely be from the carnitine present in high protein diets (Mitchell et al., 2008; Trimigno et al., 2020). In an early study conducted on choline and gut bacteria deficient rats, the amount of urinary DMA excreted was the same as control rats which indicate that choline is not an essential precursor and that intestinal bacteria were not essential for the formation of DMA (Zeisel et al., 1985). This highlights that there may be multiple pathways in mammals that allow the endogenous biosynthesis of DMA. Intermediary metabolic

pathways such as the breakdown of creatinine or the transmethylation of methylamine from sarcosine or glycine have been suggested as endogenous sources of DMA (Mitchell et al., 2008; Zeisel et al., 1985). In contrast, TMA excretion was significantly reduced in choline deficient and bacteria deficient rats, which indicates a reliance on choline and the gut microbiome (Zeisel et al., 1985).

The presence of DMA and TMA in urine have been associated with various diseases such as kidney damage, cardiovascular disease, diabetes, prostate cancer, rheumatoid arthritis and reduced bone volume (Baptista et al., 2020; Chhibber-Goel et al., 2016; Tsikas, 2020). Therefore, the increased presence of both DMA and TMA in the control diet may be as a result of the poor diet and the potential detrimental health effects thereof. These substances have not been identified in the urine of cheetahs, but TMA has been identified in the anal sac secretions of domestic cats (Banik et al., 2021). The authors concluded that cat urine has the potential to produce TMA with its distinctive "fishy" odour and this may contribute to scent marking. While both DMA and TMA have distinctive "fishy" odours and may play a role in the scent marking of cheetahs, the significantly higher concentrations on the control diet is interesting and indicates an effect by glycine. Choline chloride and dimethylglycine, which are precursors for TMA, were significantly higher in the control diet too and could be driving the increased production of TMA, but not necessarily that of DMA. Choline and dimethylglycine are also part of the glycine biosynthesis and degradation pathways and, therefore, when glycine is supplemented in the diet, it may potentially reduce the pressure on this pathway leading to a reduction in DMA, TMA and related metabolites. This could have major health benefits for the cheetah although the mechanisms are not yet clear. Importantly, the relative concentrations of DMA and TMA are very low for all three diets and, because it is not absolute, cannot be compared to literature that describes the concentration range of detrimental urinary associations to diseases.

4.3.6. Pyroglutamic acid

Pyroglutamic acid, also known as 5-oxoproline, is a cyclic amide of glutamic acid discovered in 1882 and is an intermediate metabolite in the glutathione cycle (Kumar and Bachhawat, 2012). Pyroglutamic acid is formed by the degradation of glutathione, the spontaneous cyclisation of phosphorylated glutamic acid or the degradation of proteins that contain pyroglutamic acid at the N terminus (Kumar and Bachhawat, 2012). Increased pyroglutamic acid excretion has been shown to indicate insufficient glycine availability in healthy humans after ingesting benzoic acid to deplete glycine stores (Jackson et al., 1987). Glycine was diverted to the conjugation of benzoic acid to form hippuric acid and diverted away from glutathione biosynthesis. In contrast, oral glycine supplementation in malnourished children led to decreased pyroglutamic acid excretion and increased blood glutathione concentration, which suggests that glycine is a rate limiting nutrient in growing children (Persaud et al., 1996). There is a clear link between urinary pyroglutamic acid,

glutathione biosynthesis (and therefore antioxidant status) and the role of glycine conjugation in these pathways (Meléndez-Hevia et al., 2009).

Pyroglutamic acid has previously been identified in the range of 20 to 75 µmol/L in the urine of captive cheetahs (Tordiffe and Mienie, 2019). In the current study, pyroglutamic acid was identified in the urine of all 10 cheetahs across the baseline, control and glycine supplemented diet. It contributed to the baseline diet in the PLS-DA model and was significantly lower at baseline compared to the control diet. This aligns with the abovementioned studies because the control diet would have no glycine present and, therefore, the urinary excretion of pyroglutamic acid is increased as an indicator of this deficiency. When glycine was added to their diet, the urinary excretion decreased (albeit not significantly) to levels closer to baseline. Although the cheetahs received a basic mince diet for 3 weeks prior to baseline, the low pyroglutamic acid concentration indicates the remnants of a more collagen rich diet with a potentially higher glycine content. This was also shown in Jackson et al. (1987) where individuals had different rates of glycine depletion (as indicated by increased benzoic acid ingestion) which the authors concluded may be due to the efficiency with which glycine can be mobilised for metabolic interaction. Therefore, three weeks on a sub-optimum diet would not be sufficient to naturally deplete the crucial glycine content in captive cheetahs, with four to seven weeks being the likely range as per the control diet. A four week glycine supplementation period was enough to reduce the pyroglutamic acid levels and a longer period or higher concentration may be more beneficial.

4.3.7. 1,3-Diaminopropane (1,3-DAP)

1,3-DAP is a naturally occurring polyamine along with other molecules like putrescine, cadaverine, spermidine and spermine (Moinard et al., 2005). Polyamines play a variety of physiological roles mostly in immune response such as cell growth, synthesis of proteins and nucleic acids, and cell signalling (Moinard et al., 2005). Putrescine is synthesised from ornithine by ornithine carboxylase (a rate limiting enzyme in polyamine biosynthesis) and gives rise to the other amines and their derivatives. 1,3-DAP is a close homologue of putrescine and *in vivo* acts as a repressor of ornithine carboxylase (Persson and Rosengren, 1984). The mechanisms of 1,3-DAP biosynthesis and breakdown are not well described in mammals, but in plants 1,3-DAP is synthesised from spermine and spermidine, which leads to the production of β-alanine, an important substrate for coenzyme A production (Parthasarathy et al., 2019). Increased urinary excretion of polyamines have been observed in catabolic conditions such as low calorie diets, multiple trauma and various cancers with increased plasma 1,3-DAP found in rat models of lung cancers (Liu et al., 2017; Moinard et al., 2005).

1,3-DAP was identified in the urine of all 10 cheetahs across the diets and was significantly higher at baseline compared to the control diet with the glycine diet having a similar distribution to the

control diet. Polyamines can be obtained from meat and plant based food in the diet, but also from endogenous gastrointestinal secretions, desquamation of enterocytes and bacterial synthesis (Moinard et al., 2005). While the dietary influence on 1,3-DAP, as a specific polyamine, is not clear from the literature, it may be linked to the availability of ornithine and arginine which is essential in cats (MacDonald et al., 1984; Tordiffe and Mienie, 2019). If 1,3-DAP is an inhibitor of ornithine decarboxylase in cheetahs, the higher urinary concentration at baseline may indicate a greater production of 1,3-DAP which leads to the inhibition of the polyamine pathways, as putrescine production would decrease. However, putrescine was not identified in this study and while spermidine was identified, it was fairly constant across all three diets. Additionally, the higher 1,3-DAP levels at baseline may indicate that polyamines were adequately obtained from other exogenous or endogenous sources. In contrast, the lower DAP on the control diet and glycine diet (albeit not significantly) may allow additional production of putrescine and its related metabolites in order to elicit an immune response. Due to the production of polyamines from ornithine, the lower 1,3-DAP may also be linked to the significantly higher proline (which is also produced from ornithine) in the control diet.

4.3.8. Dihydrothymine

Dihydrothymine, derived from thymine, was identified in the urine of 5 cheetahs at baseline, 6 cheetahs on the control diet and 9 cheetahs on the glycine diet. It was not identified via ANOVA in MetaboAnalyst, presumably due to the lower identification of this metabolite in the baseline and control diet, which in itself is interesting. However, when taking a multivariate approach, dihydrothymine was the greatest contributor to the diet separation in the PLS-DA model, particularly driving the baseline and glycine diet.

Dihydrothymine is formed as an intermediate after the breakdown of thymine by dihydropyrimidine dehydrogenase, ultimately forming β-aminoisobutyric acid by dihydropyrimidinase (DHP) (Löffler et al., 2005). As pyrimidines, thymine along with uracil and cytosine play an essential role in mammalian metabolism as nucleotide bases in DNA and RNA, activators in phospholipid and polysaccharide synthesis, and glycosylation of proteins and lipids (Löffler et al., 2005). There are numerous inborn errors of pyrimidine metabolism, of which high levels of urinary dihydrothymine is associated with a defect in the *DHP* gene with symptoms such as neurological abnormalities, seizures, impaired growth and gastrointestinal problems (Löffler et al., 2005; Philip et al., 2012). A single case of this condition has been reported in a malnourished stray male cat who presented with high amounts of urinary dihydrouracil and dihydrothymine, and moderate amounts of uracil and thymine, suggesting a DHP deficiency (Philip et al., 2012). In the current study, data suggests that dihydrothymine is playing a role in relation to the glycine diet compared to the baseline diet. Thymine biosynthesis leads to an equimolar glycine production of roughly 6.2 mmol/day (Meléndez-Hevia et al., 2009). Therefore, the additional glycine supplementation potentially leads to a build-

up of thymine, which leads to a greater excretion of dihydrothymine. Similarly, *de novo* biosynthesis of pyrimidines occur from glutamine in the cytosol and cheetahs have been shown to have a high urine concentration of glutamine (Tordiffe and Mienie, 2019). Although not significant, the current study demonstrated that glutamine was also an abundant metabolite showing a higher trend in the control and glycine supplemented diet compared to baseline. Glycine plays a key role in purine (such as adenine and guanine) production, which works alongside pyrimidines to produce DNA and RNA. This potentially points to a need for increased pyrimidine biosynthesis to match the purine biosynthesis (Meléndez-Hevia et al., 2009). The link between dihydrothymine and glycine is not clear, but it may be linked to excess glutamine or purine biosynthesis.

4.3.9. Methylmalonic acid (MMA)

MMA is a dicarboxylic acid that is a vital intermediate in fat and protein metabolism. Specifically, the catabolism of isoleucine, valine, methionine, and threonine as well as of odd chain fatty acids and cholesterol to propionate, ultimately leading to the entry of their metabolites into the Citric Acid Cycle (Hörster and Hoffmann, 2004). Methylmalonyl-CoA mutase (MCM) is an essential mitochondrial enzyme in this process. Defects in this enzyme lead to an accumulation of MMA in the urine and biofluids, called methylmalonic aciduria which is characterised by vomiting, lethargy, acidosis, ketosis, and coma (Hörster and Hoffmann, 2004). Vitamin B_{12} (generally obtained from dietary animal tissue) produces an essential cofactor for MCM, which catalyses the conversion of methylmalonyl-CoA to succinyl-CoA. Therefore, MMA and vitamin B_{12} have an inverse relationship often being used as diagnostic markers of vitamin B_{12} deficiency, methylmalonic aciduria and other gastrointestinal conditions (Sobczyńska-Malefora et al., 2021).

MMA was identified in the urine of all 10 cheetahs across the diets with cheetahs on the glycine supplemented diet having a significantly lower concentration than at baseline. This indicates that MCM is functioning optimally and more metabolites of the respective protein and fat metabolism are able to enter the Citric Acid Cycle for energy production. Glycine plays an important role in the conjugation of detrimental by-products resulting from the bacterial fermentation of high protein diets. This improved conjugation may lead to better absorption of vitamin B_{12} and catabolism of excess proteins to succinyl-CoA (Badenhorst et al., 2014; Sobczyńska-Malefora et al., 2021). MMA and vitamin B_{12} (along with other metabolites) have been suggested as biomarkers of gastrointestinal disease in cheetahs due to the high prevalence of these disease in this population and its success as markers in domestic cats and dogs (Fox et al., 2021). While the results of the study were largely inconclusive, it was the first study investigating potential markers in cheetahs and highlights the importance of further understanding gastrointestinal disease in cheetahs. The absolute concentrations of MMA were not determined in the current study, but the mechanism of glycine in reducing the relative concentrations warrants further investigation as this could contribute to the knowledge of their gastrointestinal health.

4.3.10. Pimelic acid

Pimelic acid (also known as α,ω-heptanedioic acid) was found in the urine of all 10 cheetahs across the baseline, control and glycine supplemented diet. A significant difference between diets was not identified by post hoc analysis, but pimelic acid contributed to the PLS-DA model specifically driving the baseline and glycine diets. Pimelic acid, a simple carboxylic acid, is the precursor for biotin (vitamin B_7) biosynthesis in microbes, fungi and plants with mammals obtaining biotin from their diet and intestinal microflora (Lin and Cronan, 2011). The biosynthesis of pimelic acid is not fully understood, but believed to be synthesised by a modified fatty acid synthetic pathway (Lin and Cronan, 2011). Biotin is an essential, water soluble vitamin that can be obtained from natural food such as meat, eggs, liver and vegetables (Zempleni et al., 2009). In the current study, biotin was not specified in the nutrient breakdown of horse meat consumed by the cheetahs, but it was provided in the supplement at 100 μg/day throughout the study (Table 1). Biotin plays a role in cell signalling, epigenetic regulation of genes, chromatin structure and as a covalently bound coenzyme for five carboxylases (Zempleni et al., 2009). Acetyl-CoA carboxylase 1 and 2 are involved in fatty acid synthesis and oxidation, and pyruvate carboxylase is a key enzyme in gluconeogenesis. Propionyl-CoA carboxylase catalyses the metabolism certain amino acids and fatty acids to the citric acid cycle and β-Methylcrotonyl-CoA carboxylase which catalyses an important step in leucine metabolism (Zempleni et al., 2009). All these pathways are crucial in energy production, with propionyl-CoA carboxylase closely linked to the discussion of MMA as it is the first enzyme to catalyse propionyl-CoA (propionate) to methylmalonyl-CoA (Hörster and Hoffmann, 2004). Therefore, increased pimelic acid may lead to increased propionyl-CoA carboxylase activation through biotin biosynthesis.

Pimelic acid has previously been identified in the urine of cheetahs at 4.5 ± 3.0 mmol/L (Tordiffe et al., 2017). At baseline, the pimelic acid concentrations were higher than both the control and glycine diet, with the difference between the baseline and control diet likely contributing to the overall significance. A greater excretion of pimelic acid may indicate sufficient biotin being obtained from the diet, and therefore a reduced need for biosynthesis of biotin from pimelic acid. On the control diet, the lower excretion may indicate the need for increased biotin biosynthesis from pimelic acid by gastrointestinal bacteria. The direct biotin consumed by the cheetahs were constant throughout the study. This finding indicates that remnants of a collagen rich diet (baseline) and glycine supplementation are influencing the production of pimelic acid or the biosynthesis of biotin by the microflora. Cats and dogs have a similar intestinal microflora to humans with changes in the microbial communities shown to be associated with acute and chronic gastrointestinal disease (Honneffer et al., 2014). Although the specific microbiome of cheetahs is still unknown, glycine may be conjugating toxic by-products influencing the production of pimelic acids and other microbial products.

4.4. Summary

A total of 151 metabolites were identified in the urine of 10 captive cheetahs at baseline, on a control diet and on a glycine supplemented diet. This was reduced to 10 metabolites that had the greatest contribution towards the metabolic differences between the diets. The change in proline, pyroglutamic acid and 1,3-diaminopropane at baseline likely reflect the influence of a historic collagen diet and not glycine supplementation. Glycine supplementation had a similar effect to the baseline diet for dimethyl sulphone, trimethylamine, dimethylamine and pimelic acid – which indicates that glycine may be responsible for the beneficial effects on the gut microbiome. On the other hand, the effects of glycine supplementation exceeded the baseline diet for fructose, dihydrothymine and methylmalonic acid. This indicates an important role of glycine in energy metabolism, gastrointestinal health and pyrimidine biosynthesis for captive cheetahs.

CHAPTER FIVE

5. SERUM METABOLITES

5.1. Introduction

Blood is the primary transporter of molecules around the body and is composed of two components – the cellular part (contains RBC, WBC and platelets) and the liquid plasma (Psychogios et al., 2011). Plasma is obtained from a blood sample when an anticoagulant is added, while serum is obtained when blood is allowed to clot and therefore the resulting supernatant contains no clotting factors (Yu et al., 2011). Both plasma and serum contain a variety of substances including proteins, nutrients, electrolytes and metabolic by-products, and serves as an indicator of all the different molecules being secreted and excreted by surrounding organs and tissues in normal and pathophysiological conditions (Psychogios et al., 2011). Due to its importance and ease of accessibility, the chemical analysis of blood has been performed for over 70 years generating normal reference ranges for many blood gases, ions and metabolites of humans and other animals. More recently, metabolomics techniques have been utilised to characterise the blood metabolome with 4229 compounds confirmed in humans (Psychogios et al., 2011). Plasma and serum metabolite analysis are both reproducible and produce equally relevant results for clinical and biological studies, although higher concentrations are found in serum (Yu et al., 2011). The metabolomics focus in animals is more limited with approximately 50 studies across different species, generally domestic animals and rodents, most of which utilised serum, plasma or urine samples (Carlos et al., 2020; Tran et al., 2020).

Although several studies have investigated a number of blood parameters in cheetahs, only two from the same laboratory have used metabolomics techniques (Tordiffe et al., 2016; Tordiffe and Mienie, 2019). The former study investigated the serum fatty acid profiles of captive and free ranging cheetahs using GC-MS. It was found that serum monounsaturated and polyunsaturated fatty acids were lower in free ranging cheetahs, likely due to the differences in dietary fat intake and composition. Tordiffe and Mienie (2019) identified 36 serum amino acids with glutamine (1624 µmol/L), alanine (772 µmol/L), arginine (670 µmol/L) and glycine (479 µmol/L) being the highest concentrations in the cheetah. These studies form the foundation of serum metabolite data for captive and free ranging cheetahs.

Glycine along with the many products related to glycine metabolic pathways have been identified in the blood of humans, pigs, rodents and birds (El Hafidi et al., 2004; Gannon et al., 2002; Imenshahidi and Hossenzadeh, 2022; Wang et al., 2014, 2013). Therefore, using a metabolomics approach to investigate the effect of glycine supplementation on the serum of cheetahs would be beneficial especially considering the high concentration of metabolically active compounds, the use of serum in nutritional studies and the limited data available on cheetah serum metabolites.

The aims of this chapter are to:

- identify and quantify (through relative concentrations) the metabolites present in the serum of 10 captive cheetahs at baseline, on a control diet and on a glycine supplemented diet;
- highlight and discuss the identified metabolites of importance.

5.2. Results

5.2.1. Identified metabolites and relative concentration

ASICS identified 60 metabolites in the serum across the 10 cheetahs in each diet. These were reduced to 48 metabolites after data reduction (20% reduction) which removed metabolites present in less than 50% of the samples in all three groups. The relative concentrations of the 20 most abundant metabolites are reported in Table 9. D-glucose was the most abundant metabolite identified in the serum across all three groups – with approximately five to six times the concentration of the next metabolite. L-glutamine, lactic acid, taurine and guanidinoacetic acid followed glucose as the most abundant for each diet. The same 20 metabolites were consistently identified across all three diets, with the only exception of L-lysine which was only abundant in the control diet. All 48 metabolites are shown in Supplementary Table S5.

Table 9. The 20 most abundant metabolites identified by NMR in the serum of 10 captive cheetahs at baseline, on a control diet and on a glycine supplemented diet

Baseline			Control diet			Glycine supplemented diet		
Metabolite	**n**	**Relative concentration**	**Metabolite**	**n**	**Relative concentration**	**Metabolite**	**n**	**Relative concentration**
D-Glucose	10	23.9 ± 2.9	**D-Glucose**	10	25.0 ± 5.4	**D-Glucose**	10	23.4 ± 3.0
L-Glutamine	10	4.9 ± 0.6	**L-Glutamine**	10	4.9 ± 0.5	**L-Glutamine**	10	4.7 ± 0.4
Lactic acid	10	3.5 ± 0.6	**Lactic acid**	10	3.7 ± 0.4	**Lactic acid**	10	3.8 ± 0.5
Taurine	10	2.7 ± 1.0	**Taurine**	10	3.5 ± 0.9	**Taurine**	10	2.9 ± 0.9
Guanidinoacetic acid	10	2.7 ± 0.6	**Guanidinoacetic acid**	10	2.9 ± 0.8	**Guanidinoacetic acid**	10	2.6 ± 0.5
D-Mannose	10	2.6 ± 0.3	**D-Mannose**	10	2.4 ± 0.3	**G-6-P**	10	2.5 ± 0.2
G-6-P	10	2.5 ± 0.3	**D-Glucuronic acid**	10	2.4 ± 0.5	**D-Mannose**	10	2.4 ± 0.3
Glyceric acid	10	2.3 ± 0.4	**G-6-P**	10	2.3 ± 0.2	**D-Glucuronic acid**	10	2.2 ± 0.4
D-Glucuronic acid	10	2.3 ± 0.4	**Glyceric acid**	9	2.3 ± 0.4	**Glyceric acid**	10	2.2 ± 0.4
D-Galactose	9	1.9 ± 0.3	**D-Galactose**	7	2.0 ± 0.3	**D-Galactose**	10	1.8 ± 0.3
L-Carnitine	10	1.8 ± 0.4	**L-Alanine**	10	1.7 ± 0.2	**L-Alanine**	10	1.7 ± 0.3

L-Alanine	10	1.7 ± 0.2	**L-Carnitine**	10	1.7 ± 0.6	**L-Carnitine**	10	1.6 ± 0.5
D-Maltose	10	1.6 ± 0.2	**D-Maltose**	9	1.6 ± 0.2	**D-Maltose**	10	1.6 ± 0.1
L-Glycine	10	1.5 ± 0.3	**L-Glycine**	10	1.5 ± 0.5	**L-Cystine**	10	1.5 ± 0.2
L-Cystine	10	1.5 ± 0.1	**L-Cystine**	10	1.5 ± 0.2	**L-Glycine**	10	1.4 ± 0.3
D-Fructose	7	1.2 ± 0.4	**D-Fructose**	7	1.5 ± 0.4	**L-Proline**	10	1.3 ± 0.2
L-Proline	10	1.2 ± 0.2	**Ethanolamine**	10	1.2 ± 0.3	**Ethanolamine**	10	1.2 ± 0.2
Ethanolamine	10	1.1 ± 0.1	**L-Proline**	10	1.2 ± 0.3	**Creatinine**	10	1.1 ± 0.2
Creatinine	10	1.1 ± 0.2	**L-Lysine**	6	1.1 ± 0.2	**D-Sorbitol**	8	1.1 ± 0.2
D-Sorbitol	9	1.0 ± 0.2	**Creatinine**	10	1.1 ± 0.1	**D-Fructose**	6	1.1 ± 0.4

Data are expressed as mean ± SD. n indicates the number of samples where the metabolite was detected. Relative concentration is an arbitrary unit. G-6-P, D-Glucose-6-Phosphate

5.2.2. Metabolites identified with univariate analysis

MetaboAnalyst did not identify any significant metabolites after FDR correction in the ANOVA. Only L-glutamic acid ($F = 6.14$, raw $p = 0.01$, adjusted $p = 0.42$) and α-aminobutyric acid ($F = 3.92$, raw $p = 0.04$, adjusted $p = 0.65$) were significant prior to the adjustment.

5.2.3. Metabolites identified with multivariate analysis

The PCA and PLS-DA plots are shown in Figure 6. PC 1 accounted for 32% variation in the serum data, while PC 2 accounted for 14%. Most of the data for all three diets are clustered, with three clear outliers – the baseline and control diet outliers represent the same individual. The 10 metabolites contributing the most to the variation of PC 1 and PC 2 are reported in Table 10. L-glycine, hypotaurine, L-carnitine, D-glucose and guanidinoacetic acid had the highest loading scores for PC 1 and, therefore, were the metabolites that had the highest weighting contribution towards the variation in PC 1. Based on the PCA plot, the metabolites contributing to PC 1 are driving most of the variation across the baseline, control and glycine diet. The metabolites contributing the most to PC 2 are lactic acid, L-threonine, L-valine, L-lysine and L-glutamine.

In the PLS-DA plot, there is a clear overlap with the baseline, control and glycine diet. This indicates poor separation of the diets in the model, which is supported by the negative Q2 values (Supplementary Figure S1) indicating that the model lacks predictive power. The discriminant variables of component 1 account for 15.4% of the variation between diets, while component 2 is 23.2%. Therefore, component 2 is a better axis for maximising the separation between diets, which

is not ideal and, again, highlights the limited predictability of this PLS-DA model. The VIP and co-efficient scores for each diet is reported in Table 10. The top VIP in component 1 and 2 were L-glutamic acid, propionic acid and α-aminobutyric acid. Similarly, L-glutamic acid had the highest co-efficient of regression for the baseline, control and glycine diets. This was followed by α-aminobutyric acid and propionic acid (baseline), taurine and ethanolamine (control), taurine and D-glucose-6-phosphate (G-6-P) (glycine supplemented).

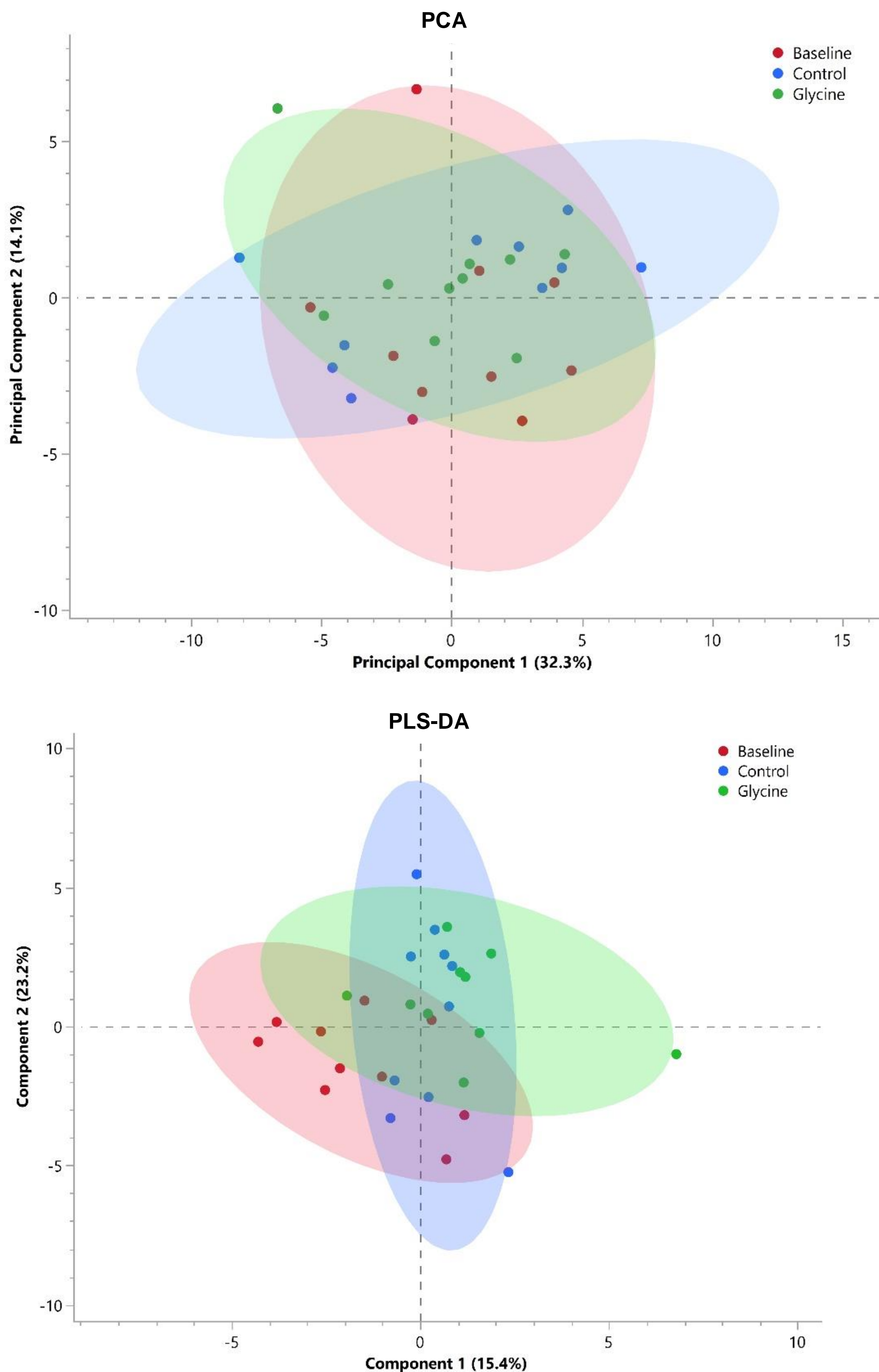

Figure 6. A two dimensional PCA (top) and PLS-DA (bottom) scores plot of the metabolites identified in the serum of 10 captive cheetahs at baseline, on the control and glycine supplemented diets. *Each point represents an individual cheetah at baseline (red), control (blue) and glycine supplemented (green) with a corresponding 95% confidence ellipse for each diet.*

Table 10. The (A) PCA loadings, (B) PLS-DA VIP scores and (C) PLS-DA diet regression coefficients for the top 10 serum metabolites in 10 captive cheetahs

A. PCA Metabolite loadings			
PC 1		**PC 2**	
L-Glycine	0.25	Lactic acid	0.29
Hypotaurine	0.25	L-Threonine	0.28
L-Carnitine	0.24	L-Valine	0.28
D-Glucose	0.24	L-Lysine	0.28
Guanidinoacetic acid	0.24	L-Glutamine	-0.27
L-Cystine	0.24	Cadaverine	-0.25
D-Glucuronic acid	0.24	Creatine	0.24
Ethanolamine	0.23	Creatinine	-0.24
N-Acetylglycine	0.22	D-Gluconic acid	-0.22
Propylene glycol	0.22	Phenethylamine	-0.18

B. PLS-DA Metabolite VIP scores			
C 1		**C 2**	
L-Glutamic acid	2.89	L-Glutamic acid	2.63
Propionic acid	2.49	Propionic acid	2.12
α -Aminobutyric acid	1.94	α-Aminobutyric acid	1.65
Lactose	1.75	Lactose	1.54
Choline chloride	1.75	Choline chloride	1.50
L-Threonine	1.61	L-Threonine	1.36
D-Mannose	1.49	D-Mannose	1.34
L-Methionine	1.46	L-Methionine	1.30
Lactic acid	1.09	Ethanolamine	1.28
L-Lysine	1.02	G-6-P	1.02

C. PLS-DA metabolite coefficients for each diet					
Baseline		**Control**		**Glycine**	
L-Glutamic acid	100.0	L-Glutamic acid	47.6	L-Glutamic acid	32.6
α-Aminobutyric acid	66.1	Taurine	40.4	Taurine	23.6
Propionic acid	63.2	Ethanolamine	35.5	G-6-P	20.8
Choline chloride	55.8	G-6-P	35.2	Ethanolamine	20.3
L-Methionine	54.9	L-Threonine	34.3	D-Maltose	19.5
Cadaverine	44.8	L-Valine	34.1	L-Threonine	18.7
Taurine	44.5	D-Maltose	33.4	L-Methionine	17.8
L-Lysine	44.1	Ascorbic acid	29.0	Propionic acid	17.6
D-Mannose	43.3	D-Galactose	28.8	L-Valine	17.5
G-6-P	41.5	D-Glucuronic acid	26.6	D-Galactose	16.1

Loadings are the weight of each metabolite and are expressed as positive or negative number depending on its direction from the PC. Coefficients are the weighted sum of the PLS-DA regression for each group. PCA, principle component analysis; PLS-DA, partial least squares discriminant analysis, VIP, variable importance in projection; PC, principle component; C, component. G-6-P, D-Glucose-6-phosphate

5.2.4. Summary of important metabolites

The 48 serum metabolites identified were reduced to 32 metabolites that were present in the top 20% of at least one component of the univariate or multivariate analysis (Supplementary Table S6). This was further reduced to seven metabolites that were present in at least four out of the eight analyses. The seven important serum metabolites were L-glutamic acid, L-threonine, α-aminobutyric acid, G-6-P, ethanolamine, L-methionine and propionic acid. The normalised concentrations of these seven metabolites are shown in Figure 7. None of the metabolites were significantly different between diets after FDR adjustment, but they still contributed to the variation

observed between the diets. α-Aminobutyric acid, G-6-P and ethanolamine were identified in all 10 cheetahs across the three diets. However, the remaining metabolites were not consistently identified across diets – most notably, L-glutamic acid was identified in nine cheetahs at baseline, three on the control diet and two on the glycine diet.

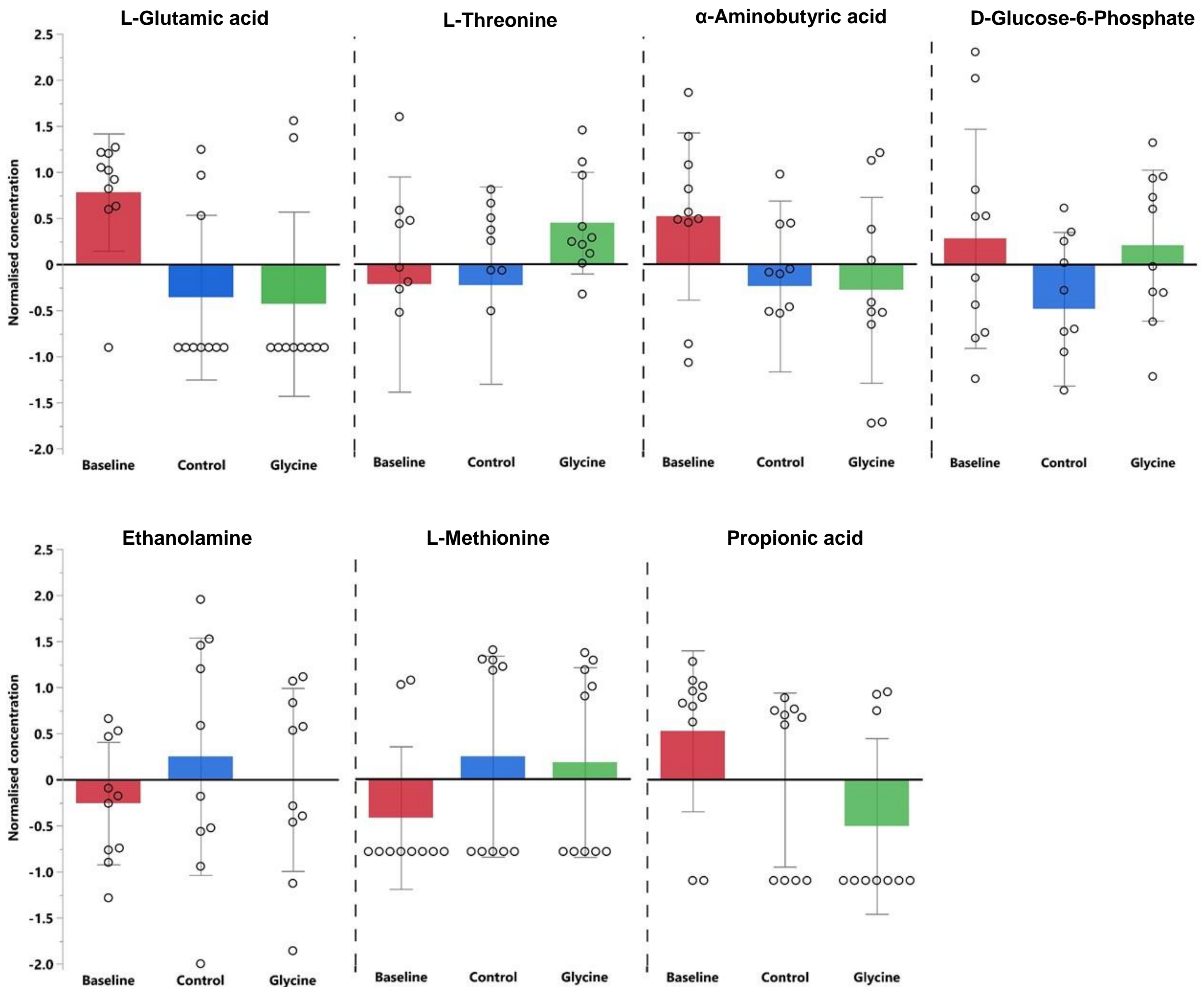

Figure 7. The normalised concentration of 7 potentially important serum metabolites identified in 10 captive cheetahs at baseline (red), on a control diet (blue) and on a glycine supplemented diet (green). *The dots represent individual values, the bar represents the mean normalised concentration and the grey line represents one standard deviation from the mean. Zero values were replaced by 20% of the lowest value during data scaling. Normalised concentrations may be a positive or negative number because it is scaled relative to the metabolite mean for all samples.*

5.3. Discussion

5.3.1. Overview of identified metabolites in serum

A total of 60 metabolites were identified in the serum of cheetahs, of which 12 were removed after data cleaning, resulting in a final number of 48 metabolites. This aligns with previous NMR data on humans where approximately 49 compounds were identified on average in human serum (Psychogios et al., 2011). Blood is the primary transporter of molecules throughout the body, and is therefore vital for homeostasis. Considering this homeostatic importance, the identification of less metabolites and the lower number of univariate significantly different metabolites in the serum of cheetahs compared to the urine, is expected. Additionally, the cross-over design allowing each cheetah to have repeated measures across the baseline, control and glycine diets and the generally conservative endogenous metabolome (which is highly responsive to external stimuli such as diet) likely contribute towards the noticeable metabolic similarities between dietary groups (Wishart, 2019). This is emphasised by the clear overlap in the PCA and PLS-DA graphs which conventionally enhances the differences or separation between groups (Figure 6).

Glucose was the most abundant metabolite identified in the serum of the cheetahs in the current study. It has previously been identified at varying concentrations in cheetahs - 6.3 ± 0.2 mmol/L (Bechert et al., 2002) and 8.5 ± 1.6 mmol/L and 11.8 ± 2.9 mmol/L with different immobilisation drugs (Buck et al., 2022). This high concentration of glucose in cheetahs aligns with what has been found using NMR in healthy humans with glucose having the second highest serum concentration (5 mmol/L) after urea (6 mmol/L) (Psychogios et al., 2011). Due to potential spectrum interference with other metabolites, urea was excluded from the spectra analysis in the current study. Glutamine was the most abundant amino acid identified in the serum, and has previously been found in the serum of cheetahs at a concentration of 1.6 ± 0.6 mmol/L (Tordiffe and Mienie, 2019).

Despite the lack of significance found using univariate methods, the PCA and PLS-DA analysis highlighted metabolites in the serum that were contributing towards potential variation between the components and diets. This analysis was used to reduce the 48 metabolites to 7 metabolites of interest which will be discussed in the sections below as well as their potential association with glycine supplementation.

5.3.2. L-Glutamic acid

Glutamic acid, often called by its stable anion glutamate, is a highly abundant amino acid with critical roles in metabolism, signalling and nutrition (Brosnan and Brosnan, 2013). Dietary glutamic acid is used almost exclusively as a metabolic fuel that is completely oxidised to CO_2 in the intestines of rats, pigs and humans (Brosnan and Brosnan, 2013). Therefore, *in vivo* biosynthesis of glutamic acid is essential and circulating glutamic acid is tightly regulated at low concentrations. Firstly, glutamic acid can be synthesised from α-ketoglutaric acid, a citric acid cycle intermediate,

by glutamate dehydrogenase and this process is reversible. Secondly, glutamic acid can be synthesised from the other "glutamate family" amino acids glutamine, arginine, proline and histidine (Brosnan and Brosnan, 2013). Along with protein synthesis, glutamic acid has many functions such as the direct production of glutathione and as a major excitatory neurotransmitter in the central nervous system (CNS) with its product γ-aminobutyric acid (GABA) being a major inhibitor (Brosnan and Brosnan, 2013; Wu, 2009). Glycine and glutamic acid are co-agonists for the N-methyl-D-aspartic acid (NMDA) receptors which are ligand gated ion channels distributed throughout the CNS for fast excitatory synaptic transmission (Yu and Lau, 2018). These receptors are needed for learning and memory and are drug targets for treating Alzheimer's disease, depression, epilepsy and schizophrenia (Yu and Lau, 2018). Indeed, serum glutamic acid concentration was higher in schizophrenic patients compared to healthy controls which may be due to an imbalance in the dopamine and glutamate systems and hypofunction of the NMDA receptors (Ivanova et al., 2014). In a recent study using HPLC, glutamate, glutamine and glycine were measured in the serum and CSF of the clinical spectrum of Alzheimer's patients (Nuzzo et al., 2021). It was found that glutamate increased in pre-clinical Alzheimer's patients compared to healthy controls, while serum glycine remained constant across the clinical spectrum. The former finding suggests that glutamate concentration tends to decrease with disease progression and that glycine may not be an ideal serum or CSF biomarker for Alzheimer's disease. Although these findings do not directly influence the current study, it indicates a link between glycine and glutamate albeit at a neurological level (Nuzzo et al., 2021; Yu and Lau, 2018).

In the current study, glutamic acid was identified in the serum of nine cheetahs at baseline, and only three and two cheetahs on the control and glycine diet, respectively. While glutamic acid was not significantly different between groups after FDR adjustment, prior to that adjustment, it was significantly higher in the baseline group compared to the control and glycine diet, likely due to the difference in identification. Glutamic acid was the biggest contributor to the VIP scores in the separation of the diets, contributing primarily to the baseline diet. Glutamic acid has previously been identified in the serum of cheetahs at a concentration of 174 ± 67 μmol/L (Tordiffe and Mienie, 2019).

Considering the conventional abundance and strict regulation of glutamic acid, it was surprising that this amino acid was not identified in most of the cheetahs on the control and glycine diet. However, it may be present at very low concentrations that were not detected with ASICS (Tardivel et al., 2017). Glutamine concentrations remained constant between the groups so it is unlikely that any change in glutamic acid is linked to a change in glutamine. Proline was the only pre-cursor to be identified in the serum, but there was no change in concentration. Conversely, proline had a significantly lower urinary excretion at baseline which may indicate greater utilisation in other pathways such as glutamate synthesis. Indeed cats lack pyrroline-5-carboxylate synthase which is needed to convert glutamic acid to proline, therefore, allowing a greater build-up of glutamic acid

or utilisation in other pathways (Wu et al. 2011). Due to the similarity between the control and glycine group, it is unlikely that any change is due to the glycine supplementation but rather the detrimental effects of a sub-optimum diet. Based on the studies in Alzheimer's and schizophrenia, the seemingly lower glutamic acid concentration may be a positive indicator of normal neurological function. Glutamic acid and glycine are required for glutathione, an important antioxidant, synthesis and reduction in glutamic acid may be an indicator of increased glutathione production due to the sub-optimum diet, even though glutathione was not identified in the cheetah serum in the current study. Pyroglutamic acid, which is an intermediate of glutathione production and is produced from glutamic acid, was highlighted as an important urinary metabolite that was significantly lower at baseline. Glutamic acid is a highly functional amino acid and the apparent difference in serum may be linked to multiple metabolic roles, such as glutathione production or energy production through α-ketoglutaric acid.

5.3.3. L-Threonine

Threonine is an essential amino acid that cannot be produced endogenously and must be obtained from the diet (Tang et al., 2021). Although not significantly different, threonine was identified at a seemingly higher concentration in the serum of ten cheetahs on the glycine supplemented diet as opposed to eight cheetahs at baseline and on the control diet, respectively. Threonine contributed more to the control and glycine diets, according to the PLS-DA model, compared to the baseline diet. Threonine plays a crucial role in lipid metabolism, protein synthesis (particularly of mucins in epithelial tissue) and maintaining intestinal health (Tang et al., 2021). Catabolism of dietary threonine occurs via three pathways. Firstly, threonine is degraded to α-ketobutyric acid and ammonia by liver threonine dehydratase which is then decarboxylated to propionyl-CoA and enters the citric acid cycle as succinyl-CoA – this is the major pathway of threonine degradation accounting for 93% of the total flux in humans (Meléndez-Hevia et al., 2009; Tang et al., 2021). Secondly, threonine dehydrogenase converts threonine to the unstable 2-amino-3-oxybutyrate which is degraded to glycine and acetyl-CoA, the latter entering the citric acid cycle for energy. This pathway only accounts for 7% of the threonine flux equating to approximately 0.3 mmol/day of glycine in adult humans (Meléndez-Hevia et al., 2009). Lastly, threonine is degraded to acetaldehyde and glycine by threonine aldolase, however, this enzyme is inactive in mammals and, therefore, this pathway is unlikely to produce glycine in the cheetah. Threonine has been identified in the serum of cheetahs at a concentration of 154 ± 67 µmol/L (Tordiffe and Mienie, 2019).

In broiler chickens, supplemented glycine has been shown to reduce the activity of all three catabolic threonine enzymes (threonine aldolase, threonine dehydrogenase and threonine dehydratase) increasing the amount of available threonine for physiological processes (Bernardino et al., 2011). In a study by Ospina-Rojas et al. (2013), growing broiler chickens on a low protein diet were supplemented with different concentrations of glycine and serine (which is a precursor for

glycine biosynthesis) and digestible threonine. Higher concentrations of glycine and serine led to improved growth which was enhanced by greater threonine supplementation suggesting that threonine may reduce the requirement for dietary glycine. Increasing levels of glycine and serine also increased intestinal mucosa secretion, which may directly be attributed to the glycine-rich central domain of mucin or indirectly due to the improved integrity of the gastrointestinal tract by preventing threonine degradation. Reducing threonine degradation may result in reducing the formation of volatile fatty acids (e.g. acetic acid, propionic acid, and butyric acid) by commensal bacteria, therefore, improving intestinal function (Tang et al., 2021). This indicates that glycine supplementation may be essential for the re-synthesis of mucin, which has a high intestinal turnover, thereby supporting the direct role of threonine in mucin production (Ospina-Rojas et al., 2013). In thoroughbred colts and mares that received threonine supplementation for five days, plasma glycine concentration was significantly higher on the supplemented diet compared to the cross-over control diet which likely reflects the catabolism of threonine to glycine (Mastellar et al., 2016). These studies highlight the connection between glycine and threonine, especially regarding mucin biosynthesis.

Although the higher relative concentration of the glycine supplemented diet was not significantly different to the control or baseline, the trend in the current study supports what has been found in earlier studies. As threonine can only be obtained from the diet, the only direct source for the cheetahs would be the horse meat consumed which would contain approximately 0.84 g threonine/100g meat (Table 1). The amount of meat consumed in the current study remained fairly constant and, therefore, glycine supplementation may have a sparing effect on threonine. This would then allow more threonine to be directed towards propionyl-CoA for energy in the citric acid cycle or towards intestinal mucin production which would improve gastrointestinal health. Although the threonine catabolic enzyme activities were not investigated in the current study, a reduction in their respective activities may account for the increase in threonine with glycine supplementation, as was reported for broiler chickens (Bernardino et al., 2011). Overall, an increase in threonine concentration has been associated with improved gastrointestinal health, which may be crucial for cheetahs in captivity.

5.3.4. α-Aminobutyric acid (AABA)

ABBA is a non-proteinogenic amino acid synthesized from methionine and threonine degradation to α-ketobutyric acid (Chiarla et al., 2011). α-Ketobutyric acid can either be converted to AABA or decarboxylated to propionic acid to enter the citric acid cycle (Chiarla et al., 2011; Tang et al., 2021). The well-known isomer of AABA is GABA, which is a major inhibitory neurotransmitter in the CNS (Wang et al., 2020). Although the exact function and pathways of ABBA are poorly understood, it has been suggested as a biomarker in many conditions including severe sepsis, liver disease, osteoporosis and COVID-19 in humans (Atila et al., 2021; Chiarla et al., 2011; Wang et al., 2020).

In mice, it was found that AABA increases intracellular glutathione production through AMPK, therefore, regulating oxidative stress defence (Irino et al., 2016).

ABBA has previously been identified in the serum of cheetahs at 22 ± 12 µmol/L (Tordiffe and Mienie, 2019). In the current study, ABBA was identified at low relative concentrations in the serum of all 10 cheetahs across diets. Prior to adjustment, significance was found between diets in which the baseline diet appeared to be higher than the control and glycine supplemented diets. ABBA had a high VIP score for both component 1 and 2, which indicates its contribution to the PLS-DA model. The amount of threonine and methionine consumed by the cheetahs would have been consistent for all three diets, and the similarity between the control and glycine groups indicate that glycine supplementation may not be directly affecting the production of ABBA. In sepsis patients, there was a positive correlation between high ABBA concentrations and increasing glycine concentrations (Chiarla et al., 2011). During the conversion of choline to glycine, homocysteine utilises a methyl group to convert to methionine – this indicates a link between a glycine synthesis pathway and ABBA production from methionine (Wang et al., 2013). Additionally, glycine can be synthesised from threonine which can also produce ABBA. Therefore, ABBA could potentially increase or decrease depending on whether the endogenous or exogenous glycine is utilised, respectively. This could attribute to the consistent concentrations for the control and glycine diets. However, the baseline diet which likely contains a wider diversity of amino acids may lead to a greater production of ABBA. This metabolite appears to be an indicator of the catabolism of methionine (for which cats have a high demand) and threonine, although studies have also suggested ABBA as an indicator for glutathione production, and does not appear to have a clear functional role especially regarding glycine supplementation.

5.3.5. D-Glucose-6-Phosphte (G-6-P)

G-6-P was identified in the serum of all 10 cheetahs at baseline, on the control and glycine diet. It contributed towards the baseline and control diet PLS-DA models, and indeed appears to have a higher relative concentration at baseline compared to the control diet. G-6-P is an important saccharide formed when glucose, from the blood, enters the cell (such as hepatocytes) and is immediately phosphorylated to G-6-P to prevent the diffusion of free glucose out of the cell (Rajas et al., 2019). G-6-P has many intracellular fates including glycolysis, pentose phosphate pathway, glycogen synthesis and glucose production (Rajas et al., 2019). For carnivores, such as domestic cats and cheetahs, there is a close link between protein and glucose metabolism because the former provides substrates that channel into gluconeogenic pathways (Schermerhorn, 2013). Unlike other mammals, cats lack glucokinase and it is hypothesized that the conversion of intracellular glucose to G-6-P is driven by hexokinase I (Schermerhorn, 2013). Hexokinase has kinetic features which make it an unsuitable replacement for glucokinase, largely due to its inhibition by its product, G-6-P, which is continuously being produced by gluconeogenesis in feline

hepatocytes. Therefore, it is believed that there is a “pull effect” where G-6-P is being redirected away from gluconeogenic pathways and towards glycogen biosynthesis. This enhances G-6-P flux and reactivates hexokinase which allows an increase in glucose phosphorylation (Schermerhorn, 2013). The efficient phosphorylation of hepatic glucose is an area that requires more research.

G-6-P does not appear to be regularly identified in the blood of humans or animals, with glucose and G-6-P dehydrogenase or related enzymes being the target of investigations. Whether this is due to its primarily intracellular role or simply because it is not considered a conventional indicator of glucose metabolism, is not apparent. However, in a recent study the serum concentrations of various simple sugars and their derivatives were investigated in Humboldt penguins using GC-MS (Schaeffer et al., 2020). G-6-P was identified as a key sugar in discriminating between different nesting habitats presumably due to different energy requirements. In the current study, the seemingly higher G-6-P concentrations at baseline followed by the glycine diet, may be due to the gluconeogenic role of glycine particularly with pyruvate production (Wang et al., 2013). The serum glucose concentration remained constant across diets, although the glucose excretion was significantly higher on the control diet compared to the baseline and glycine diet indicating higher production on the control diet. This indicates an inverse relationship between glucose and G-6-P which aligns with its interconversion. Additionally, glycine is directly involved in the biosynthesis of creatine which is important for rapid energy production and glycine supplementation has been shown to reduce glucose production and insulin sensitivity (Imenshahidi and Hossenzadeh, 2022; Wang et al., 2013). Therefore, glycine supplementation may promote other pathways of energy production such as creatine and glycolysis, and reduce the need for endogenous glucose production. This reduced pressure may decrease the flux through the glucose and G-6-P pathways which leads to a build-up of G-6-P that further inhibits the activity of hexokinase. The hepatic enzyme activities were not measured in this study and may provide a useful indicator of the carbohydrate metabolism of the cheetah.

5.3.6. Ethanolamine

Ethanolamine is an organic compound that is abundantly present in the cell membranes of mammalian cells primarily as a key component of the phospholipid, phosphatidylethanolamine (PE) (Zhou et al., 2017). Ethanolamine is an essential nutrient obtained from dietary lipids, the turnover of enterocytes and bacterial cells, and indirectly via the decarboxylation of phosphatidylserine (PS) to PE resulting in a base exchange between serine and ethanolamine. PE and phosphatidylcholine (PC) account for more than half of the total phospholipid content in eukaryotic cells and, as a result, play an important role in cellular structure and function (Zhou et al., 2017). PE is produced via the CDP-ethanolamine pathway in the endoplasmic reticulum in which ethanolamine is phosphorylated by ethanolamine kinase ultimately producing PE. Along with PE production, ethanolamine can also be catabolised by intestinal bacteria producing many products including acetaldehyde, ammonia,

acetyl CoA, ethanol and acetic acid – most of which (excluding acetyl CoA) are harmful to the intestine and microflora (Zhou et al., 2017). Therefore, ethanolamine plays a role in regulating lipid metabolism directly and indirectly though the intestine microflora, as well as increasing the concentration of short chain fatty acids (acetic and butyric acid) which are end products of bacterial fermentation.

In the current study, ethanolamine was identified in the serum of all cheetahs across the three diets and is seemingly higher in the control diet compared to the baseline, which is also reflected by its contribution to the control diet in the PLS-DA model. Captive cheetahs would generally consume a high protein and moderate fat diet with horse meat providing an estimated 6 to 12% lipid composition (Badiani et al., 1997; Bechert et al., 2002). In an early rat model, it was found that glycine is not directly converted to ethanolamine but is converted indirectly via serine as an intermediate (Greenberg and Harris, 1950). As the cheetahs would receive the same amount of fat throughout the study, differences would likely be linked to the higher collagen at baseline or glycine on the glycine diet. However, the concentration of ethanolamine was seemingly higher on the control diet – which may be due to the turnover of intestinal cells and bacteria. In domestic cats, 144 serum metabolites were identified that were related to lipid metabolism with decreasing concentrations of ethanolamine derivatives associated with early weight loss (Pallotto et al., 2021). Interestingly, glycine derivatives (such as sarcosine and N-acetylglycine) increased and decreased at various stages of weight loss, but was associated throughout the 16 week study. In the current study, cheetahs had significantly lower weight on the glycine diet which may account for the lower ethanolamine concentration and the effect that glycine has on adiposity, insulin sensitivity and β-oxidation (El Hafidi et al., 2004). However, there was no significance and baseline appeared to have a lower concentration even though the cheetahs weighed more. The utilization of ethanolamine by gut bacteria has been shown to be beneficial for their survival in the host with this cycle leading to greater proliferation of epithelial cells ultimately producing more ethanolamine and its products (Zhou et al., 2017). This may account for the higher concentration on the control diet, whereby more bacteria are utilizing ethanolamine leading to increased proliferation and turnover of epithelial cells and bacteria. Although acetic acid and other phospholipid metabolites were not identified, the notably identification of ethanolamine indicates a link between lipid metabolism, the biosynthesis and degradation of ethanolamine by the gut microbiome and the importance of dietary collagen and glycine.

5.3.7. L-Methionine

Methionine was identified at low concentrations in 2 cheetahs at baseline, and 5 cheetahs on the control and glycine supplemented diet. The lower identification at baseline is likely driving its contribution. Methionine, the sulphur containing essential amino acid, has previously been identified in the serum of cheetahs at a concentration of 63 ± 28 μmol/L (Tordiffe and Mienie, 2019).

Methionine must be obtained from dietary protein and is necessary for normal growth and development in mammals as it is the precursor for S-adenosylmethionine (SAM, which is utilised for the biosynthesis of the polyamines spermidine and spermine), homocysteine, succinyl-CoA and phosphatidylcholine (phospholipid) (Finkelstein, 1990). Homocysteine is an important substrate in the recycling of intracellular folates, the catabolism of choline and betaine; and the biosynthesis of cystathionine, cysteine and glutathione (Finkelstein, 1998). The conversion of available methionine to SAM via methionine adenosyltransferase is the major pathway for methionine metabolism and is produced at higher concentrations in the liver compared to other tissues (Finkelstein, 1990).

Cats have a high dietary demand for methionine and cysteine due to its gluconeogenic role, along with all the other functions (MacDonald et al., 1984). In a recent study investigating the metabolic differences between domestic cats at varying risks for diabetes, it was found that serum methionine was significantly higher in Maine Coon cats which have moderate risk for diabetes compared to the low risk Birman cat (Mu et al., 2021). Interestingly, increased dimethylglycine was also found in the Maine Coon, which is a precursor for glycine synthesis and also the product formed during the conversion of homocysteine to methionine. In domestic shorthair cats fed a high choline diet, the plasma methionine concentration was the only amino acid that was significantly higher – likely due to the increased betaine and re-methylation of homocysteine (Verbrugghe et al., 2021). Reduced plasma methionine and metabolites of the methionine cycle have also been shown to be associated with longevity when the metabolic profile of species with different lifespans were investigated (Mota-Martorell et al., 2021). These studies indicate that methionine concentration appears to change according to different breeds or species and conditions. The seemingly higher (or more identified) methionine concentration on the control and glycine diets compared to the baseline in the current study, may be due to the length of time on a diet and the dietary change, and not specifically the effect of glycine supplementation. However, the routes of increased methionine concentration may be different as the pathway of endogenous glycine biosynthesis from choline does lead to methionine formation. Alternatively, glycine supplementation may reduce pressure on glutathione production and allow the recycling of homocysteine to methionine. Methionine plays an extensive role in the body and concentrations may vary in the cheetah according to different dietary conditions, like in the domestic cat, but the direct link to glycine supplementation is unclear.

5.3.8. Propionic acid

Propionic acid, or propionate, was identified at low concentrations in the serum of 8 cheetahs at baseline, 6 cheetahs on the control diet and 3 cheetahs on the glycine supplemented diet. The low identification was likely due to the low relative concentrations of this metabolite in the serum. Despite this, propionic acid had a high VIP score and noticeably contributed towards the baseline diet. Propionic acid is formed by the fermentation of undigested food (mostly starch and fibre, but also proteins and long chained fatty acids) by the intestinal microbiota, and along with acetic acid

and butyric acid is grouped as a short chain fatty acid (SCFA) (Al-Lahham et al., 2010). These substrates are broken down and converted via several potential pathways to pyruvate which is ultimately converted to propionic acid and propionyl-CoA, where the latter enters the citric acid cycle as succinyl-CoA (Al-Lahham et al., 2010).

Cheetahs on a beef based diet have been shown to have higher faecal ammonia, indole, phenol and SCFA concentrations compared to other exotic captive felids (Vester et al., 2008). In a similar study comparing the differences between horse and beef based diets in captive cheetahs, the beef diet resulted in two to three times higher concentrations of all the aforementioned products (Vester et al., 2010). In both studies, acetic acid (~70%) was the SCFA with the highest concentration followed by propionic acid (~22%) and butyric acid (~8%). Additionally, on a rabbit carcass diet cheetahs produced lower concentrations of SCFA and putrefactive compounds, which the authors suggest is due to the beneficial effects of poorly digestible animal tissue such as hair and bones (Depauw et al., 2013). In the current study, propionic acid was identified more frequently in the baseline diet compared to the control diet with the glycine diet having the lowest identified relative concentration. These cheetahs were fed horse meat which, based on the study by Vester et al. (2010), would produce lower concentrations of SCFA compared to another meat diet – but interestingly the baseline diet which would likely contain remnants of a more collagen rich and optimum diet had the highest concentration. This supports what was proposed by Depauw et al. (2011) that the poorly digestible animal tissue is causing the beneficial SCFA effects and not necessarily any metabolic benefits that a historic collagen rich diet may provide. In a short report, it was found that patients with propionic acidaemia had normally lower plasma glycine (664 μmol/L) compared to times when they experienced severe episodes of hyperammonaemia and acidosis (1200 μmol/L). The authors suggested that this may be due to increased glycine conjugation of propionic acid (forming propionylglycine) during periods of acidosis (Al-Hassnan et al., 2003). Indeed, in the current study, glycine supplementation may lead to increased conjugation of propionic acid and other detrimental intestinal products which results in lower concentrations, therefore, improving gastrointestinal health. Acetic and butyric acid were not identified in the serum so it is difficult to draw conclusions relating to the SCFA, but glycine may be beneficial for those acids too.

5.4. Summary

In summary, 60 metabolites were identified in the serum of ten cheetahs at baseline, on a control and glycine supplemented diet. This was reduced to 7 metabolites of interest that contributed most notably to the metabolic differences, based on multivariate analyses, between cheetahs in the three groups. Glutamic acid, α-aminobutyric acid and methionine contributed primarily to the baseline diet and likely do not reflect specific changes to glycine supplementation. However, threonine,

glucose-6-phosphate, ethanolamine and propionic acid concentrations were influenced by glycine supplementation. These metabolites play various roles in energy pathways, glucose metabolism, lipid metabolism and intestinal microbiome fermentation. Specifically, the increased threonine and decreased propionic acid concentrations on the glycine supplemented diet reflect positive changes in the gastrointestinal tract.

CHAPTER SIX

6. GENERAL DISCUSSION AND CONCLUSION

Cheetahs are a unique and vulnerable felid species, currently only occupying 9% of their global historic range resulting in captivity being an important tool for their conservation (Durant et al., 2017). However, in captivity they are prone to an array of diseases that are not seen in their free ranging counterparts such as chronic lymphoplasmacytic gastritis, glomerulosclerosis and veno-occlusive disease (Munson, 1993; Munson et al., 1999). Various factors of captivity, namely stress, lack of physical activity and diet have been suggested as possible causes of the increased disease prevalence. To date, no studies have investigated the link between physical activity and disease and the evidence regarding the effects of stress is somewhat inconsistent. Therefore, diet has become an increasingly important avenue of investigation over the last 20 years. This is particularly important when considering the contrast of feeding in the wild, in which cheetahs would capture small to medium prey and consume entire carcasses, compared to feeding in captivity, in which cheetahs would generally consume raw muscle meat (with limited carcass components) and vitamin and mineral supplementation (Hayward et al., 2006; Whitehouse-Tedd et al., 2015). Incomplete digestion of this high meat protein diet may lead to increased bacterial fermentation in the intestine elevating the production of putrefactive compounds such as SCFA, ammonia, phenolic and indolic compounds (Depauw et al., 2013). Carcass components such as hair, skin and bone have been shown to be poorly fermentable substrates and, therefore, may act as a bulking agent filling up the intestines and forming a barrier between the bacteria and substrates (Depauw et al., 2013, 2012a). Additionally, captive cheetahs have been shown to produce more detrimental compounds compared to other captive felids (Vester et al., 2008). This indicates a link between the captive diet and the unique metabolism and physiology of the cheetah.

The major difference between the captive and wild diet lies in the presence of carcass components such as cartilage, bones, ligaments, tendons and skin – all of which are rich in collagen. Glycine is the most abundant amino acid present in collagen, which may indicate a deficiency of this amino acid in a captive diet (Wang et al., 2013). Indeed, a third of the most abundant organic acids excreted in the urine of cheetahs are associated with glycine conjugates which indicates a substantial need for dietary glycine (Tordiffe et al., 2017). Therefore, the role of glycine in collagen biosynthesis and in the conjugation of organic acids which allows the excretion of detrimental compounds, is particularly important when the gut microbiome and gastrointestinal health of captive cheetahs is considered. Glycine has many other physiological roles in the body such as the biosynthesis of creatine, glutathione, purines, haem and as a neurotransmitter which may be beneficial for captive cheetahs as has been shown in rodents, pigs and humans (Imenshahidi and Hossenzadeh, 2022; McCarty et al., 2018; Wang et al., 2013).

Therefore the purpose of this study was to investigate the metabolic effects of glycine supplementation in captive cheetahs fed a raw muscle meat diet. A metabolomics approach using urine and serum was utilised in order to obtain a holistic idea of the many metabolites and pathways involved. This was used alongside body measurements, haematology and serum biochemistry.

An overview of the important metabolites identified in the urine and serum, their potential pathways and the link to glycine are displayed in Figure 8. Although only 10 urine and 7 serum metabolites were discussed previously, Figure 8 shows the 31 important metabolites identified in the urine and 18 metabolites identified in the serum (the list of metabolites are included in Supplementary Table S4 and S6). Due to the large number of metabolites identified in the urine, only those that were identified with both univariate and multivariate analyses were included in this figure – this approach reduced 65 important metabolites to 31 metabolites. For serum, the metabolites identified with more than one analyses were included in the overview figure. Only maltose, valine and taurine overlapped in the urine and serum. This figure shows changes in metabolite concentration in relation to the control diet (not necessarily just univariate significant differences) and, therefore, highlights three potential influences. Firstly, the effect of a more collagen rich diet represented by the baseline diet (red box). It is clear from the previous chapters that in several metabolites the baseline diet is eliciting a response beyond that of glycine supplementation. This may be due to the historic or remnant effect of a collagen diet containing high levels of glycine or potentially due to other aspects of a carcass diet such as the indigestible components. Secondly, the effect of glycine supplementation alone (green box) and, thirdly, the matched effect of a collagen diet or a glycine supplemented diet (yellow box).

In previous chapters, the metabolites and the effect of glycine was described individually and not as a collective pathway. However, based on Figure 8 it is clear that there is notable overlap and connections between the identified metabolites with four specific pathways highlighted: (1) methionine associated pathways, (2) threonine associated pathways, (3) carbohydrate and fat metabolism, and (4) glutamic acid associated pathways. Each of these are linked to specific physiological functions of glycine namely collagen biosynthesis, glutathione production, creatine and energy production, DNA and RNA biosynthesis, and digestion and conjugation. These functions are highlighted by different colour areas in the figure. The discussion below will focus on each of these pathways. For ease of reference, it would be beneficial to move outwards from methionine and threonine when looking at Figure 8 as they are the starting points of the discussion. However, the figure is interconnected and can be read from any starting point.

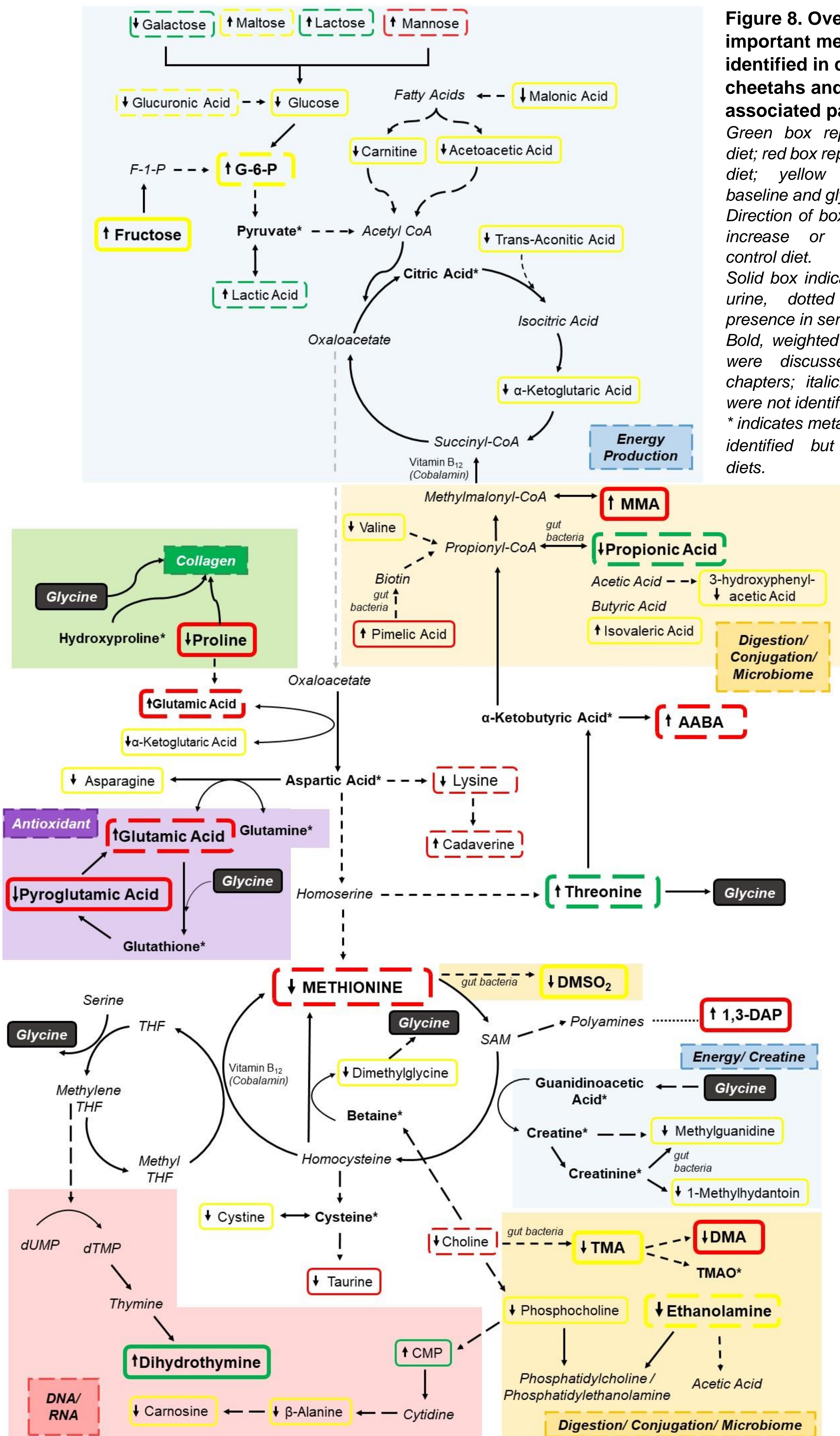

Figure 8. Overview of the important metabolites identified in captive cheetahs and their associated pathways.

*Green box represents glycine diet; red box represents baseline diet; yellow box represent baseline and glycine diet. Direction of box arrow indicates increase or decrease from control diet. Solid box indicates presence in urine, dotted box indicates presence in serum. Bold, weighted box metabolites were discussed in previous chapters; italicised metabolites were not identified in this study; * indicates metabolites that were identified but similar across diets.*

G-6-P, glucose-6-phosphate; F-1-P, fructose-1-phosphate; MMA, methylmalonic acid; AABA, α-aminobutyric acid; DMSO₂, dimethyl sulphone; 1,3-DAP, 1,3-diaminopropane; SAM, S-adenosyl methionine; THF, tetrahydrofolate; TMP, thymidine monophosphate; UMP, uridine monophosphate; CMP, cytidine monophosphate

6.1. Methionine associated pathways

Methionine is an essential amino acid with a high dietary demand in cats, although cats are less tolerant of excess methionine compared to other amino acids indicating a high utilisation in the body (MacDonald et al., 1984). In the study by Tordiffe and Mienie (2019), methionine was identified at low concentrations relative to other amino acids in the urine and serum of cheetahs. This was also found in the current study with the baseline diet having a lower concentration in both the serum and urine compared to the control and glycine diet, although the concentration was low across all diets. Despite these low concentrations, methionine is clearly a hub of metabolism with many important metabolites identified in this study forming downstream substrates or products of methionine pathways. In terms of the gut microbiome, the lower methionine concentration at baseline led to lower production and excretion of $DMSO_2$ produced from methionine degradation (He and Slupsky, 2014). Similarly, TMA (and subsequently DMA) concentrations were also lower, likely due to the lower concentration of its precursor's choline, dimethylglycine and carnitine. Interestingly, glycine supplementation also led to lower concentrations of both $DMSO_2$ and TMA despite methionine and choline only being lower at baseline. This may indicate that glycine supplementation is affecting the production of $DMSO_2$ and TMA through the reduction of other sources (such as carnitine and dimethylglycine). Additionally, glycine in itself may be acting as a substrate leading to the reduced utilisation of other amino acids and producing different products, or that the bacterial population of a collagen and glycine rich diet is similar allowing the same bacteria to survive and produce similar products (Dai et al., 2011). In a recent study by Baptista et al. (2020), $DMSO_2$ and TMA had a strong association with bone turnover and volume, and although the mechanisms are not understood, it supports what was found in the current study with both metabolites being influenced by dietary collagen and glycine, which would also have a strong link to bone. Overall, glycine appears to be providing the same gut bacterial benefits as a more collagen rich diet.

The conversion of methionine to SAM is the major methionine catabolic pathway (Finkelstein, 1990). SAM transfers a propylamine moiety to putrescine or spermidine to yield spermidine or spermine which are important polyamines. While the exact route of 1,3-DAP biosynthesis is unclear, it is a close homologue of putrescine and, therefore, may also be linked to SAM along with ornithine (Moinard et al., 2005). Polyamines are involved in cell proliferation, signalling and overall immune response, and although no other polyamine was significantly identified in the current study, the higher concentration of 1,3-DAP on the baseline diet alone indicates a collagen effect (or another factor of the pre-study diet such as other poorly digestible components) that is not necessary replicated with glycine.

The subsequent conversion of SAM to homocysteine allows the corresponding conversion of guanidinoacetic acid to creatine through the donation of a methyl group (Wyss and Kaddurah-Daouk, 2000). Glycine is directly involved in this process as the transfer of the amidino group of

arginine to glycine yields ornithine and guanidinoacetic acid which represents the first step of creatine biosynthesis (Wyss and Kaddurah-Daouk, 2000). The reversible conversion of creatine to phosphocreatine via creatine kinase is crucial for rapid ATP production, both of which can spontaneously be degraded to creatinine which is excreted from the body. Creatine and creatinine can also be converted by gut bacteria to methylguanidine which is a uremic toxin, and along with other guanidino compounds (such as creatinine and creatine) could have neurotoxic effects if allowed to accumulate in the body (Falconi et al., 2021; Wyss and Kaddurah-Daouk, 2000). Similarly, 1-methylhydantoin can also be produced from the degradation of creatinine by gut bacteria and is either taken up by the body or degraded further by bacteria producing sarcosine which can lead to glycine biosynthesis (Wyss and Kaddurah-Daouk, 2000). Although guanidinoacetic acid, creatine and creatinine were identified in the current study, the concentrations were similar across diets. However, methylguanidine and 1-methylhydantoin were both significantly lower on the baseline and glycine diets compared to the control diet. This in itself may have positive health implications for the cheetah, but it may also indicate that more creatine is being produced from the collagen and glycine rich diets allowing the interconversion to phosphocreatine thereby producing more ATP. In the wild, cheetahs require rapid energy production in order to chase and capture prey, and this finding indicates that a collagen or glycine rich diet (which would be reflected in their wild diet) may provide them with this crucial high energy phosphate to replenish ATP and also protect them from detrimental metabolites formed during the bacterial degradation of creatine and creatinine. Despite the lower physical activity experienced in captivity, these effects would undoubtedly still be beneficial for other metabolic processes.

Homocysteine, which is a key substrate produced from SAM, is involved in several important biological processes (Finkelstein, 1998). Firstly, homocysteine allows the re-synthesis of methionine through the utilisation of betaine as a methyl donor forming dimethylglycine. This is the only route for the degradation of betaine which is an intermediate in choline catabolism. Dimethylglycine ultimately forms glycine and accounts for approximately 1.4 mmol/day glycine in humans (Meléndez-Hevia et al., 2009). The lower concentrations of dimethylglycine on the baseline and glycine diet indicates that this route is not utilised to the same extent when exogenous glycine is provided or when collagen concentrations are high. Additionally, choline concentrations were lower on the baseline diet which would indicate decreased catabolism to betaine, however on the glycine diet choline may be re-directed towards phosphocholine biosynthesis. The second important homocysteine methylation reaction, which also converts to methionine, allows the recycling of 5-methyltetrahydrofolate (methyl-THF) to THF which constitutes the folate cycle (Finkelstein, 1998). Crucially, THF to methylene-THF via glycine hydroxymethyltransferase allows the biosynthesis of glycine from serine – this is the major pathway for endogenous glycine biosynthesis in mammals accounting for 34 mmol/day glycine in humans (Meléndez-Hevia et al., 2009). Therefore, these two pathways highlight the close connection between glycine biosynthesis

and the methionine cycle. Lastly, homocysteine can be irreversibly converted to cysteine and finally taurine. The decreased concentrations of taurine and cystine (oxidised cysteine) on the baseline and baseline and glycine diet, respectively, indicates lower flux through this pathway when exogenous glycine is provided. This is potentially due to reduced homocysteine degradation, due to reduced flux though the methionine cycle as the need for endogenous glycine is reduced, or potentially homocysteine redirection towards SAM production or the folate cycle.

Phosphocholine, produced from choline, was lower in the baseline and glycine diets which would indicate a decreased production of PC. Similarly, ethanolamine was an important metabolite highlighted in the serum of the baseline and glycine diets with decreased concentrations leading to decreased PE production as well as PC production through the CDP-ethanolamine pathway (Zhou et al., 2017). Together, it appears that cheetahs on the collagen and glycine rich diets have a lower source of phospholipids. This may also be due to the close link between lipid metabolism and the gut microbiome, in which the turnover of enterocytes and bacterial cells is a rich lipid source that can be converted to ethanolamine and PE (Zhou et al., 2017). The utilisation of ethanolamine by gut bacteria allows their survival and growth, therefore a lower concentration may indicate reduced bacterial colony growth and, along with the potential reduced biosynthesis of acetic acid, may indicate improved gastrointestinal health. Interestingly, CMP, which can be produced from phosphocholine, had a higher urinary concentration in the glycine diets. This highlights the redirection from choline towards cytidine biosynthesis, a pyrimidine component of RNA. β-alanine can be produced via several pathways, including from gut bacteria, but Figure 8 is highlighting the potential production from cytidine to uracil, ultimately producing β-alanine (Tiedje et al., 2010). β-alanine is a neuromodulator and can potentially be considered as a small molecule neurotransmitter with glycine and GABA binding sites. The largest concentration of β-alanine in the body is found as carnosine, formed from β-alanine and histidine, which can act as a neuroprotective agent and as an intramuscular pH buffer (Tiedje et al., 2010). The concentration of carnosine from biosynthesis is directly proportional to the concentration of β-alanine, therefore, the reduced concentration of both these metabolites on the baseline and glycine diets potentially indicates a decrease in the catabolism of pyrimidines. This is echoed by the higher concentrations of dihydrothymine, a derivative of the pyrimidine thymine, which was higher in the glycine diet and can be formed from the folate cycle using methylene-THF and, therefore, has a close relationship with glycine biosynthesis from serine as thymine biosynthesis leads to equimolar glycine production (Meléndez-Hevia et al., 2009). The endogenous and exogenous source of glycine may be leading to this potential increase in thymine (and subsequently dihydrothymine biosynthesis). Although glycine is known to play a direct role in purine biosynthesis, this data also suggests a close relationship with pyrimidine biosynthesis as downstream products of glycine biosynthesis. Pyrimidines and purines form the base of DNA and RNA and, therefore, play an essential role in the body with defects in these processes being fatal for mammals (Löffler et al., 2005). In this study, the relative

concentrations of the identified pyrimidine metabolites were low and changes are unlikely to indicate any fatal effects on cellular processes, but it does indicate that the glycine component of a collagen diet may be the major contributor towards the biosynthesis of these crucial compounds in cheetahs.

6.2. Threonine associated pathways

Threonine is an essential amino acid in cats and its degradation is a minor source of glycine in humans (Meléndez-Hevia et al., 2009). The increased concentration of threonine on the glycine diet is likely due to the exogenous glycine reducing the amount of endogenous glycine required by threonine degradation, allowing the redirection of threonine elsewhere. This could lead to increased mucosal protein synthesis which accounts for approximately 70% of threonine utilisation (which is not depicted in Figure 8) and would have intestinal benefits (Tang et al., 2021). It may also lead to increased flux towards α-ketobutyric acid and propionyl-CoA. Therefore, glycine supplementation may have a sparing effect on threonine, simulating dietary threonine supplementation which has been shown to be beneficial in rodents, broilers and pigs (Tang et al., 2021).

At baseline, there is an increased conversion of α-ketobutyric acid to AABA which is not replicated by the glycine diet. The function of AABA is still largely unknown. Therefore, this would lead to increased production of propionyl-CoA on the glycine diet. Unfortunately, the concentration of propionyl-CoA was not determined in this study, but the concentration of propionic acid may be used as an indicator. Propionic acid was lower on the glycine diet which may indicate increased propionyl-CoA. Similarly, the increased urinary MMA concentrations at baseline (with glycine being significantly lower compared to baseline) indicates that glycine may be influencing the flux towards propionyl-CoA and methylmalonyl-CoA towards succinyl-CoA, and not necessarily the remnants of a collagen rich diet. The increased concentration of pimelic acid on the baseline diet (and to a lesser extent on the glycine diet) may indicate a greater production of biotin which is a coenzyme for propionyl-CoA carboxylase that catalyses the conversion of propionyl-CoA to methylmalonyl-CoA (Zempleni et al., 2009). Alternatively, biotin could be used as a coenzyme in other pathways. There are a number of additional sources of propionyl-CoA including valine, isoleucine and odd chain fatty acids, all of which may be influencing the concentration. The reduced concentration of by-products of this pathway on the glycine diet may indicate greater flux towards the citric acid cycle. On the other hand, the baseline diet may also be leading to greater flux through these pathways but is producing increased by-products such as MMA and propionic acid (which may be detrimental). The effect of the baseline diet on propionyl-CoA is not likely to be caused by changes in threonine but potential other routes of propionyl-CoA production.

Propionic acid was the only SCFA identified in this study, but the identification of 3-hydroxyphenylacetic acid (decreased) and isovaleric acid (increased) may be indicative of other SCFAs. Despite the structural similarity between acetic acid and 3-hydroxyphenylacetic acid, it may

not be linked to SCFA production. Nonetheless, it likely has a microbial origin as has been found with 4-hydroxyphenylacetic acid in cheetahs (Tordiffe et al., 2017). The reduced concentration of propionic acid on the glycine diet in the current study supports what was found by Depauw et al. (2013), but it also highlights that the effect of glycine specifically leading to a decreased concentration and not necessarily the long lasting remnants of a collagen diet. Alternatively, the actual carcass tissue components may elicit the same response, but that would be over a shorter period and cannot be concluded from this study. Isovaleric acid (a BCFA) has previously been identified in cheetahs by Depauw et al. (2013) in which the concentration was lower, along with all the other SCFAs and BCFAs, on a carcass diet compared to a minced beef diet. The increased concentration of isovaleric acid in the current study does not match what was found previously, although this BCFA contributes a very small percentage compared to the total SCFA (Depauw et al., 2013; Vester et al., 2010). Overall, considering that all cheetahs consumed a similar amount of muscle meat throughout the study, the reduced production of products of protein digestion and fermentation on the glycine diet compared to both the baseline and control diet, indicate a positive gastrointestinal effect of glycine. This may be due to an increase in threonine allowing intestinal mucosa production, thereby improving intestinal integrity, as well as improved glycine conjugation of organic acids and other detrimental products formed during digestion and fermentation of the high protein diet consumed by captive cheetahs.

6.3. Carbohydrate and fat metabolism

As obligate carnivores, the primary source of glucose in cheetahs is from proteins that link into gluconeogenic pathways (Schermerhorn, 2013). The identification of mannose, lactose, maltose and galactose as important metabolites in the serum is somewhat surprising considering the very low carbohydrate ingestion in cheetahs, especially with the sole consumption of horse muscle meat which would have no carbohydrates (Badiani et al., 1997). While no carbohydrate requirements have been shown for cats, they are generally able to efficiently utilise most common carbohydrates added to their diets (MacDonald et al., 1984). Interestingly, glucose and galactose covalently bind to collagen, and connective tissue also contains glycosaminoglycans – this may account for the slightly higher concentrations of these sugars on the baseline diet (Depauw et al., 2012a). All these identified sugars contain glucose monomers and, therefore, they may contribute towards the glucose concentration in the cheetah. Despite these potential contributions, the excretion of glucose was lower in the baseline and glycine diets. However, this may be due to increased conversion to G-6-P and flux through glycolysis. There is also a decreased production of glucuronic acid, which is a derivative of glucose, on the glycine and baseline diet which may also highlight flux through other pathways. Fructose concentration was higher in the glycine and baseline diet compared to the control diet, which may be contributing to the increased G-6-P concentration. This G-6-P could then lead to increased pyruvate and increased acetyl-CoA production to enter the citric acid cycle.

The slightly higher lactic acid on the glycine diet may indicate increased flux through glycolysis (producing lactic acid from pyruvate). The concentrations of pyruvate remained low and fairly constant between diets, which also supports that any increase in glycolysis caused by glycine leading to pyruvate would be quickly converted to lactic acid or acetyl-CoA. Glycine supplementation in humans has been shown to reduce glucose concentrations and improve insulin sensitivity, and therefore has recently been suggested as a potential therapeutic treatment for obesity and diabetes (Imenshahidi and Hossenzadeh, 2022). This may be similar in cheetahs with lower glucose found on a collagen rich and glycine diet, despite the generally higher concentrations of potential glucose sources (such as mannose, lactose and maltose). This may be due to increased flux through glycolysis indicated by the increased G-6-P concentrations, although few intermediates of this pathway were identified. The similarities between the glycine and baseline diet also indicate that glycine is a major contributor and not simply the historic effects of collagen, although the presence of connective tissue which binds glucose and may act as a source of carbohydrates in cheetahs could lead to increased glucose when cheetahs are fed a carcass diet.

Malonic acid, along with its coenzyme malonyl-CoA, is involved with fatty acid biosynthesis. No fatty acids were identified in this study. Therefore, the lower concentration of malonic acid on the baseline and glycine diet may provide an indication of reduced fatty acid biosynthesis. Although cats and cheetahs have reduced capacity to synthesise certain fatty acids (such as linoleic acid), it is challenging to draw any conclusions around fatty acid biosynthesis and ingestion from this current study (Bauer, 1997). Carnitine transports fatty acids across the mitochondrial membrane to undergo β-oxidation and increased circulating and urine concentrations have been found in cats with feline hepatic lipidosis, but whether this shift is appropriate in magnitude for the metabolic disturbance, is unclear (Verbrugghe and Bakovic, 2013). In the current study, carnitine was lower on the baseline and glycine diet which may indicate reduced transport due to reduced fatty acids. The ketone body, acetoacetic acid, was also lower on the baseline and glycine diets. This may be a result of reduced utilisation of fatty acids and ketone bodies and reduced β-oxidation. Increasing acetoacetic acid concentrations was found in obese cats during a 16 week weight loss period, indicating a conversion to ketone bodies for energy rather than storing fats as triglycerides (Pallotto et al., 2021). The reduced concentrations of malonic acid, carnitine and acetoacetic acid on the baseline and glycine diets indicate a reduced synthesis, transport and oxidation of fatty acids. This may simply be indicative of a healthy body weight in contrast to what has been observed in obese cats and cats with hepatic lipidosis (Pallotto et al., 2021; Verbrugghe and Bakovic, 2013). In terms of body weight, the cheetahs in the current study had a significant decrease in weight from the baseline to glycine diet, which may be linked to fat loss, although both diets appeared to have a similar response to fat metabolism as highlighted by the abovementioned metabolites. Therefore, the increased weight loss may be linked to the role of glycine in the biosynthesis of bile salts as well as its role in the conjugation to bile acids improving excretion from the body – these are important for

the digestion and absorption of dietary fat (Meléndez-Hevia et al., 2009; Wang et al., 2013). Markers of bile acid metabolism, such as cholate, decreased with weight loss in cats which may be due to the role of bile acids in lipid digestion but also as signalling molecules that regulate metabolism and inflammation (Pallotto et al., 2021).

α-Ketoglutaric acid was the only significant metabolite identified that is directly involved in the citric acid cycle. The concentration was lower in the baseline and glycine diet, which is likely due to its involvement in glutamic acid biosynthesis (which will be discussed in the next section). Trans-aconitic acid, which is an intermediate linked to citric acid and isocitric acid, was also lower in the baseline and glycine diets. It is difficult to draw any conclusions regarding the citric acid cycle, but it appears that a historic collagen diet and a glycine diet may be causing similar effects. These effects may be shifting the entry points of compounds into the citric acid cycle, causing increased flux through succinyl-CoA and acetyl-CoA (from pyruvate) which was described earlier. This would then allow other metabolites, such as α-ketoglutaric acid and oxaloacetate, to be re-directed towards other functions such as glutamic acid production and aspartic acid production while still maintaining appropriate flux and producing ATP. Overall, it appears that a collagen and glycine-rich diet elicited similar responses to carbohydrate and fat metabolism with reduced glucose production, increased flux through glycolysis, reduced fatty acid biosynthesis and β-oxidation. It also appears that energy production from the citric acid cycle may be shifted towards succinyl-CoA and acetyl-CoA (from pyruvate) to balance the redirection of oxaloacetate and α-ketoglutaric acid towards aspartic acid and glutamic acid production.

6.4. Glutamic acid associated pathways

The conversion of oxaloacetate, from the citric acid cycle, to aspartic acid allows the corresponding reversible conversion of α-ketoglutaric acid to glutamic acid (Brosnan and Brosnan, 2013). Similarly, glutamic acid can also be produced from proline, although this conversion is not reversible as cats lack pyrroline-5-carboxylate synthase (Wu et al., 2011). Glutamic acid had a high concentration on the baseline diet which corresponds with the lower concentration of proline and α-ketoglutaric acid, indicating a greater conversion to glutamic acid. Glycine, proline and hydroxyproline collectively make up the vitally important structural protein, collagen (Wang et al., 2013). Therefore, the lower urinary proline excretion on the baseline diet likely indicates a reduced turnover of collagen due to higher collagen ingestion and maintenance, while the opposite is seen on the control diet which is collagen and glycine deficient. The excretion of hydroxyproline was also slightly higher in the control group, although it was not identified as significant. Together, these indicate that the control group had a higher collagen turnover due to collagen deficiency, and has also been found in young cheetahs with potential nutritional deficiencies (Tordiffe and Mienie, 2019). Neither glycine nor any of its derivatives or conjugates were identified as important across the analyses. It is apparent that the baseline diet, which still contains remnants of a collagen rich

diet, has the greatest influence and that any impact that glycine supplementation may be having directly on collagen is minimal. It is interesting that despite the 3 week habituation period where no carcass components other than muscle meat were consumed by the cheetahs, there is still a clear metabolic influence on collagen that cannot be replicated by 4 weeks of glycine supplementation. This could be due to the collagen effect build up over a long period of time (cheetahs received mixed carcass components for at least 6 months prior to the study) or potentially the re-direction of glycine towards other physiological functions and not collagen biosynthesis. The latter could also emphasise that 30 g of glycine per kg meat (which would equal between 30 g to 60 g per serving) provided in this study is insufficient when the diet is completely collagen deficient. Additionally, this could also indicate that the baseline contains higher levels of glycine than would be expected.

Aspartic acid can also lead to lysine biosynthesis which degrades to the odorous amine cadaverine. Despite the lower lysine concentration at baseline, the baseline diet produced a higher amount of cadaverine. Cadaverine derivatives were previously identified at high concentration in urine of cheetahs, which the authors suggested may be due to the high muscle meat intake (Tordiffe et al., 2017). In the current study, the muscle meat content remained the same, but surprisingly the control and glycine diet yielded a lower cadaverine concentration. This may indicate that a collagen deficient diet (independent of glycine deficiency) redirects aspartic acid towards homoserine and methionine metabolism and away from lysine and asparagine. The decreased cadaverine concentration may be beneficial for the cheetah.

Glutamic acid can also be formed from glutamine, which occurs during the conversion of aspartic acid to asparagine. Asparagine excretion was significantly lower in the baseline and glycine groups, which may indicate that the high glutamic acid concentration on the baseline diet is primarily a result of proline and α-ketoglutaric acid. Although not highlighted in this study, urinary glutamine was slightly higher on the control and glycine diets which supports the lower glutamic acid found on those diets. Glutamic acid, glycine and cysteine are substrates required to produce the major antioxidant glutathione (Brosnan and Brosnan, 2013). Glycine and cysteine were similar across diets, while the higher glutamic acid concentration at baseline likely indicates a greater production of glutathione. This is reflected by the lower concentrations of pyroglutamic acid. This potential increased glutathione production would protect against oxidative damage and convey an improved immunity– this may explain the higher eosinophil and basophil WBC count in the control and glycine groups. Additionally, the lower pyroglutamic acid also indicates that there is adequate glycine as they have an inverse relationship (Jackson et al., 1987). This supports the above theory that there was indeed a glycine deficiency on the control diet that was not fully corrected by glycine supplementation and may lead to a decreased biosynthesis of glutathione, which would be detrimental. Overall, it appears that collagen (or historic metabolic effects of collagen) has a greater impact on glutamic acid related pathways than glycine alone and that when glycine is limited, it

appears to be redirected to other pathways in cheetahs, such as pathways that promote glycine biosynthesis.

6.5. Study limitations and future directions

There were several limitations of this study, mostly linked to challenges around the study design and data analysis. Firstly, the two groups were not split equally and the length of the control diet was not the same between groups – this was purely due to logistical challenges. Secondly, the habituation period was implemented to standardise the diets for all cheetahs and it was anticipated that the 3 week habituation would also lead to a collagen and glycine deficiency. While the former occurred, the latter likely did not occur as it is apparent from the data throughout the study that the baseline diet had effects that, on many occasions, exceeded that of the glycine supplementation. This indicates that the cheetah's diet prior to the start of the study had much higher levels of collagen and glycine than was anticipated. Ideally, samples could have been collected before the habituation period which would have been a better representation of a collagen diet. As no intervention studies of this nature have been conducted in cheetahs, the length of the diet intervention (i.e. 4 weeks) and the amount of glycine (i.e. 30 g per kg meat) was deemed suitable based on studies in rodents, pigs and humans and fell within the time and funding constraints of the study. However, this may not have been long enough nor at a sufficient concentration to elicit a metabolic response similar to a collagen diet. Nevertheless, this is a realistic amount that could be implemented in captive facilities over an extended period of time.

Lastly, there were limitations associated with the NMR analysis and the ASICS software package. NMR inherently creates complex spectra in which it can be challenging to identify individual metabolites that are contributing to the spectra. The use of ASICS, as an automated software, was a simple and reliable approach to this challenge. However, ASICS has two notable limitations. Firstly, it contains a library of 190 metabolites and, therefore, only metabolites within this library could be identified in the current study. As a result, there may be a large number of metabolites that were not identified in the urine and serum of cheetahs. Secondly, the concentrations obtained were relative to the spectrum and not an absolute concentration. This has no direct implications to the data as all comparisons were made between study groups, but does limit comparison to the literature especially those using similar approaches in cats and cheetahs. These are not unique problems as all studies utilising NMR would face similar challenges.

Future studies should investigate the metabolic response of wild or captive cheetahs to a carcass diet which would provide an important nutritional reference. It may also emphasise aspects of metabolism that are influenced by the carcass diet and allow functional and feasible nutritional strategies to be implemented in captive facilities, especially when frequent carcass component diets are not possible. Future studies could also investigate the effect of glycine over an extended period

of time (such as six months) or the effects in a cheetah population with gastrointestinal diseases, as the current study was performed using a healthy and young cohort. Additionally, the present study has used an untargeted approach to highlight key areas and it may be beneficial to use a targeted approach and alternative separation and identification techniques (such as GC-MS) to enhance the data obtained. For example, targeted approaches could focus specifically on methionine, threonine, energy metabolism or organic compounds from bacterial fermentation as highlighted in the current study. Other molecular techniques such as such as fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR) and sequencing (454-pyrosequencing and shotgun sequencing) may also be beneficial to further investigate the gut microbiome in captive cheetahs as has previously been conducted in domestic cats (Lyu et al., 2020). This approach would be particularly helpful when investigating their acute and long-term response to dietary changes (such as glycine supplementation) for three main reasons: (i) the microbiome rapidly responds to short-term macronutrient changes; (ii) long-term dietary habits are a major determinant in the bacterial composition of an individual's gut microbiota; and (iii) dietary changes have varying effects in different individuals (Lyu et al., 2020).

6.6. Conclusion

This was the first study to investigate the metabolic effects of glycine supplementation in captive cheetahs in an attempt to further understand nutrition and health in captivity. It appears that glycine supplementation had a direct effect on threonine and methionine related pathways, specifically that of threonine sparing, pyrimidine biosynthesis and decreasing the production of bacterial fermentation products which may prove to be extremely beneficial in improving gastrointestinal health. Glycine supplementation had a limited effect on collagen and glutathione production, indicating that when cheetahs experience glycine or collagen deficiency that glycine is redirected to other pathways potentially those that promote glycine biosynthesis or other metabolic pathways. Along with glycine, this study also highlighted the historic or remnant effect of a collagen rich diet which would be achieved through the consumption of bones, skin and connective tissue. This effect was similar to glycine supplementation for energy metabolism, specifically that of creatine production, carbohydrate and fat metabolism. Changes to these pathways, in particular glycolysis and succinyl-CoA biosynthesis, may indicate a shift that takes place when collagen or glycine is deficient in cheetahs. Additionally, these latter findings indicate that the effects of a collagen rich diet can be maintained in cheetahs for up to 3 weeks even with no additional sources of glycine and collagen, and that depletion only occurs between 3 to 7 weeks. The findings of this study indicate the diverse metabolic effects of glycine and collagen in captive cheetahs and future studies should use a targeted approach to further elaborate on these pathways. Glycine supplementation may be a useful tool to improve the health of captive cheetahs especially when dietary collagen is limited.

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APPENDIX 1

Supplementary Tables

Table S1. ASICS reference library of 190 metabolites

Chemical Name	Chemical Name in PubChem	PubChem ID	CAS ID
1-Methyl-L-Histidine	1-Methyl-L-histidine	92105	332-80-9
1-Methylhydantoin	1-Methylhydantoin	69217	616-04-6
1,3-Diaminopropane	1,3-diaminopropane	428	109-76-2
2-AminoAdipicAcid	2-Aminohexanedioic acid	469	542-32-5
2-AminobutyricAcid	DL-2-Aminobutyric acid	6657	2835-81-6
2-Deoxyadenosine	2'-deoxyadenosine	13730	958-09-8
2-Deoxycytidine	2'-deoxycytidine	13711	951-77-9
2-Deoxyguanosine	2'-deoxyguanosine	187790	961-07-9
2-HydroxybutyricAcid	2-Hydroxybutyric acid	11266	600-15-7
2-HydroxyphenylAceticAcid	2-Hydroxyphenylacetic acid	11970	614-75-5
2-MethylglutaricAcid	2-Methylglutaric Acid	12046	1115-81-7
2-Oxobutyrate	2-oxobutanoic acid	58	600-18-0
2-Oxoglutarate	2-ketoglutaric acid	51	328-50-7
2-Oxoisovalerate	3-Methyl-2-oxobutyric acid	49	759-05-7
2-PicolinicAcid	Picolinic acid	1018	98-98-6
2-Propanol	Isopropanol	3776	67-63-0
3-Hydroxybutyrate	3-hydroxybutyric acid	441	300-85-6
3-HydroxyphenylAceticAcid	3-Hydroxyphenylacetic acid	12122	621-37-4
3-Methyl-L-Histidine	3-Methyl-L-histidine	64969	368-16-1
3-MethyladipicAcid	3-Methyladipic Acid	12292	03-01-3058
3-Methylxanthine	3-Methylxanthine	70639	1076-22-8
3-PhenylPropionicAcid	Hydrocinnamic acid	107	501-52-0
4-AminoHippuricAcid	4-Aminohippuric acid	2148	61-78-9
4-EthylPhenol	4-Ethylphenol	31242	123-07-9
4-HydroxyphenylAceticAcid	4-hydroxyphenylacetic acid	127	156-38-7
5-AminoValericAcid	5-Aminovaleric acid	138	660-88-8
7-Methylxanthine	7-Methylxanthine	68374	552-62-5
Acetaminophen	Acetaminophen	1983	103-90-2
AceticAcid	Acetic acid	176	64-19-7
Acetoacetate	Acetoacetic acid	96	541-50-4
Acetone	Acetone	180	67-64-1
Adenine	Adenine	190	73-24-5
Adenosine	Adenosine	60961	58-61-7
AdipicAcid	Adipic acid	196	124-04-9
ADP	Adenosine 5'-diphosphate	6022	58-64-0
Allantoin	Allantoin	204	97-59-6
alpha-HydroxyisobutyricAcid	2-Hydroxyisobutyric acid	11671	594-61-6
AMP	Adenosine 5'-monophosphate	6083	61-19-8
ArgininosuccinicAcid	Argininosuccinic acid	16950	2387-71-5
AscorbicAcid	Ascorbic acid	54670067	50-81-7
ATP	Adenosine triphosphate	5957	56-65-5
Azelaic Acid	Azelaic acid	2266	123-99-9

BenzoicAcid	Benzoic acid	243	65-85-0
Beta-Alanine	Beta-alanine	239	107-95-9
beta-HydroxyisovalericAcid	beta-Hydroxyisovaleric acid	69362	625-08-1
Betaine	Betaine	247	107-43-7
Butyrate	Butyric acid	264	107-92-6
Cadaverine	1,5-Diaminopentane	273	462-94-2
CDP	Cytidine 5'-diphosphate	6132	63-38-7
CholineChloride	Choline chloride	6209	67-48-1
CitraconicAcid	Citraconic acid	643798	498-23-7
Citrate	Citric acid	311	77-92-9
CMP	5'-cytidylic acid / Cytidine monophosphate	6131	63-37-6
Creatine	Creatine	586	57-00-1
Creatinine	Creatinine	588	60-27-5
CTP	Cytidine triphosphate	6176	65-47-4
Cytosine	Cytosine	597	71-30-7
D-Fructose	D-Fructose	5984	57-48-7
D-Fucose	6-Deoxyhexopyranose	840	3615-37-0
D-Galactose	D-Galactose	6036	59-23-4
D-GluconicAcid	Gluconic acid	10690	526-95-4
D-Glucose	D-Glucose	5793	50-99-7
D-Glucose-6-Phosphate	Glucose 6-phosphate	5958	299-31-0
D-GlucuronicAcid	D-Glucuronic acid	65041	03-12-6556
D-Maltose	Maltose	6255	69-79-4
D-Mannose	D-Mannose	18950	530-26-7
D-Sorbitol	D-Sorbitol	5780	50-70-4
dAMP	2'-deoxyadenosine-5'-monophosphate	12599	653-63-4
DehydroAscorbicAcid	Dehydroascorbic acid	440667	490-83-5
Dihydrothymine	Dihydrothymine	93556	696-04-8
Dimethylamine	Dimethylamine	674	124-40-3
Dimethylglycine	N,N-dimethylglycine	673	1118-68-9
Dimethylsulfone	Dimethyl sulfone	6213	67-71-0
Ethanolamine	Ethanolamine	700	141-43-5
EthylmalonicAcid	Ethylmalonic acid	11756	601-75-2
Formate	Formic acid	284	64-18-6
FumaricAcid	Fumaric acid	444972	110-17-8
GABA	4-aminobutyric acid	119	56-12-2
Galactitol	Galactitol	11850	608-66-2
GDP	Guanosine 5'-diphosphate	8977	146-91-8
GlutaconicAcid	Glutaconic acid	5280498	628-48-8
GlutaricAcid	Glutaric acid	743	110-94-1
GlycericAcid	DL-Glyceric acid	752	473-81-4
Glycerol	Glycerol	753	56-81-5
Glycerophosphocholine	SN-glycero-3-phosphocholine	657272	28319-77-9
Glycogen	Glycogen	439177	9005-79-2
GlycolicAcid	Glycolic acid	757	79-14-1
GlyoxylicAcid	Glyoxylic acid	760	298-12-4
GMP	5'-Guanylic acid / guanosine monophosphate	6804	85-32-5

GTP	Guanosine triphosphate	6830	86-01-01
GuanidinoaceticAcid	Glycocyamine	763	352-97-6
HippuricAcid	Hippuric acid	464	495-69-2
HomovanillicAcid	Homovanillic acid	1738	306-08-1
Hypotaurine	Hypotaurine	107812	300-84-5
Hypoxanthine	Hypoxanthine	790	68-94-0
IMP	Inosinic acid	8582	131-99-7
Indoxylsulfate	Indoxyl sulfate	10258	487-94-5
Inosine	Inosine	6021	58-63-9
Isobutyrate	Isobutyric acid	6590	79-31-2
IsocitricAcid	Isocitric acid	1198	320-77-4
IsovalericAcid	Isovaleric acid	10430	503-74-2
KynurenicAcid	Kynurenic acid	3845	492-27-3
L-Alanine	L-alanine	5950	56-41-7
L-Anserine	Anserine	112072	584-85-0
L-Arabitol	L-arabitol	439255	7643-75-6
L-Arginine	L-arginine	6322	74-79-3
L-Asparagine	L-asparagine	6267	70-47-3
L-Aspartate	L-aspartic acid	5960	56-84-8
L-Carnitine	L-carnitine	10917	541-15-1
L-Carnosine	L-Carnosine	439224	305-84-0
L-Citrulline	L-citrulline	9750	372-75-8
L-Cysteine	L-cysteine	5862	52-90-4
L-Cystine	L-cystine	67678	56-89-3
L-GlutamicAcid	L-glutamic acid	33032	56-86-0
L-Glutamine	L-glutamine	5961	56-85-9
L-Glutathione-oxidized	Oxiglutatione	65359	27025-41-8
L-Glutathione-reduced	Glutathione	124886	70-18-8
L-Glycine	Glycine	750	56-40-6
L-Histidine	L-histidine	6274	71-00-1
L-Isoleucine	L-isoleucine	6306	73-32-5
L-Leucine	L-leucine	6106	61-90-5
L-Lysine	L-lysine	5962	56-87-1
L-Methionine	L-methionine	6137	63-68-3
L-Ornithine	L-ornithine	6262	70-26-8
L-Phenylalanine	L-phenylalanine	6140	63-91-2
L-Proline	L-proline	145742	147-85-3
L-Serine	L-serine	5951	56-45-1
L-Threonine	L-threonine	6288	72-19-5
L-Tryptophane	L-tryptophan	6305	73-22-3
L-Tyrosine	L-tyrosine	6057	60-18-4
L-Valine	L-valine	6287	72-18-4
Lactate	Lactic acid	612	50-21-5
Lactose	Lactose	440995	63-42-3
Levoglucosan	Levoglucosan	2724705	498-07-7
LevulinicAcid	Levulinic acid	11579	123-76-2
MalicAcid	Malic acid	525	6915-15-7
Malonate	Malonic acid	867	141-82-2

MandelicAcid	Dl-Mandelic acid	1292	90-64-2
Methanol	Methanol	887	67-56-1
Methylamine	Methanamine	6329	74-89-5
Methylguanidine	Methylguanidine	10111	471-29-4
MethylmalonicAcid	Methylmalonic acid	487	516-05-2
Myo-Inositol	Scyllo-inositol	892	87-89-8
N-(2-Furoyl)Glycine	N-(2-Furoyl)glycine	21863	5657-19-2
N-Acetyl-L-AsparticAcid	N-Acetyl-L-aspartic acid	65065	997-55-7
N-Acetylglycine	N-Acetylglycine	10972	543-24-8
NAD	Nadide	5892	53-84-9
NADP	NADP	5886	53-59-8
NicotinicAcid	Nicotinic acid	938	59-67-6
NicotinuricAcid	Nicotinuric acid	68499	583-08-4
O-Acetyl-L-Carnitine	Acetyl-L-carnitine	7045767	3040-38-8
Oxypurinol	Oxypurinol	4644	2465-59-0
PantothenicAcid	Pantothenic acid	6613	79-83-4
Phenethylamine	Phenethylamine	1001	64-04-0
PhenylglyoxylicAcid	Benzoylformic acid	11915	611-73-4
Phosphocholine	Phosphocholine	1014	107-73-3
PimelicAcid	Pimelic acid	385	111-16-0
Propionate	Propionic acid	1032	79-09-4
PropyleneGlycol	Propylene glycol	1030	57-55-6
Pyrocatechol	Pyrocatechol	289	120-80-9
PyroglutamicAcid	L-Pyroglutamic acid	7405	98-79-3
Pyruvic-Acid	Pyruvic acid	1060	127-17-3
QuinolinicAcid	Quinolinic acid	1066	89-00-9
S-Acetamidomethylcysteine	S-(Acetamidomethyl)-L-cysteine	1590100	19647-70-2
SaccaricAcid	D-glucaric acid	33037	87-73-0
Sarcosine	Sarcosine	1088	107-97-1
SebacicAcid	Sebacic acid	5192	111-20-6
Spermidine	Spermidine	1102	124-20-9
Succinate	Succinic acid	1110	110-15-6
SyringicAcid	Syringic acid	10742	530-57-4
TartaricAcid	DL-Tartaric acid	875	133-37-9
Taurine	Taurine	1123	107-35-7
Threitol	D-Threitol	169019	2418-52-2
ThreonicAcid	Threonic acid	151152	14-12-3909
TMAO	Trimethylamine oxide	1145	1184-78-7
trans-4-Hydroxy-L-Proline	trans-4-Hydroxy-L-proline	5810	51-35-4
Trans-AcotinicAcid	Trans-Aconitic acid	444212	4023-65-8
trans-FerulicAcid	Ferulic acid	445858	537-98-4
Trigonelline	Trigonelline	5570	535-83-1
Trimethylamine	Trimethylamine	1146	75-50-3
UDP	Uridine 5'-diphosphate	6031	58-98-0
UDPG	UDP-glucose	8629	133-89-1
UMP	Uridine 5'-monophosphate / Uridine monophosphate	6030	58-97-9
Uracil	Uracil	1174	66-22-8
Uridine	Uridine	6029	58-96-8

UrocanicAcid	Urocanic acid	736715	104-98-3
UTP	Uridine 5'-triphosphate	6133	63-39-8
Valerate	Valeric acid	7991	109-52-4
VanillicAcid	Vanillic acid	8468	121-34-6
Xylitol	Xylitol	6912	87-99-0

Table S2. Relative concentration of the 112 metabolites identified in the urine of captive cheetahs at baseline, on a control and glycine supplemented diet (n=10).

Baseline Diet				Control Diet				Glycine Diet			
Metabolite	**n**	**Mean**	**SD**	**Metabolite**	**n**	**Mean**	**SD**	**Metabolite**	**n**	**Mean**	**SD**
Creatinine	10	64.11	47.37	**Creatinine**	10	72.76	37.56	**Creatinine**	10	56.90	46.17
Glycolic Acid	4	21.53	29.15	**Allantoin**	10	13.73	2.91	**Allantoin**	10	15.19	3.77
Allantoin	10	14.26	3.18	**Malonate**	10	13.23	4.91	**L-Cysteine**	10	10.07	1.62
L-Cysteine	10	10.46	0.74	**TMAO**	10	10.17	7.17	**Glycolic Acid**	7	9.59	6.24
Malonate	10	7.84	4.74	**L-Cysteine**	10	9.86	0.88	**Malonate**	10	9.57	4.04
L-Ornithine	10	6.12	1.33	**Glycolic Acid**	4	9.48	5.90	**TMAO**	10	7.31	4.66
Guanidinoacetic Acid	4	5.13	4.40	**L-Carnitine**	10	7.32	2.64	**Guanidinoacetic Acid**	7	6.81	5.23
L-Proline	10	4.54	0.39	**Guanidinoacetic Acid**	5	6.72	5.47	**L-Ornithine**	10	6.38	1.15
DHA	10	4.30	1.08	**L-Ornithine**	10	5.77	0.97	**L-Proline**	10	5.12	0.46
L-Carnitine	10	3.87	2.27	**L-Proline**	10	5.50	0.70	**L-Carnitine**	10	4.96	2.22
TMAO	10	3.87	3.59	**Taurine**	10	4.42	1.36	**DHA**	10	4.09	0.79
1,3-DAP	10	3.35	0.33	**DHA**	9	3.95	0.91	**Taurine**	10	3.82	1.09
L-Tryptophane	8	3.06	0.61	**Choline Chloride**	10	3.75	1.24	**L-Glutamine**	10	3.22	0.77
L-Arginine	10	3.05	0.50	**Phosphocholine**	10	3.52	0.88	**Choline Chloride**	10	3.15	1.09
Taurine	10	3.03	1.10	**2-Oxoglutarate**	10	3.41	0.35	**1,3-DAP**	10	3.05	0.31
Choline Chloride	10	2.99	1.09	**L-Glutamine**	10	3.35	1.14	**L-Arginine**	10	2.98	0.34
2-Oxoglutarate	10	2.70	0.42	**D-Glucose**	10	3.23	0.58	**2-Oxoglutarate**	10	2.93	0.29
D-Maltose	10	2.61	0.41	**D-Maltose**	10	3.20	0.48	**L-Tryptophane**	10	2.80	0.61
D-Glucose	10	2.59	0.35	**L-Arginine**	10	3.19	0.68	**Phosphocholine**	10	2.79	0.71
L-Lysine	9	2.58	0.35	**Dimethylglycine**	10	3.18	2.14	**D-Maltose**	10	2.56	0.58
Phosphocholine	10	2.56	1.02	**Creatine**	7	3.07	2.76	**Threonic Acid**	9	2.55	0.73
L-Citrulline	10	2.55	0.22	**1,3-DAP**	10	2.96	0.34	**L-Lysine**	8	2.53	0.40
L-Glutamine	10	2.53	0.89	**Threonic Acid**	8	2.73	1.01	**D-Glucose**	10	2.52	0.60
D-Galactose	9	2.48	1.12	**L-Tryptophane**	9	2.72	0.55	**β-Alanine**	10	2.51	0.29
β-Alanine	10	2.40	0.26	**β-Alanine**	10	2.72	0.19	**L-Citrulline**	10	2.45	0.27
D-Gluconic Acid	8	2.38	0.78	**D-Gluconic Acid**	7	2.64	0.97	**Glycerophospho-choline**	10	2.06	0.49
ThreonicAcid	9	1.99	0.68	**1-Methylhydantoin**	10	2.61	2.28	**Phenethylamine**	10	1.85	0.34
Glycerophospho-choline	10	1.87	0.55	**L-Citrulline**	10	2.58	0.35	**Glycogen**	10	1.83	0.29
Phenethylamine	10	1.84	0.45	**L-Asparagine**	9	2.57	1.08	**Lactose**	10	1.78	0.77
Glycogen	10	1.76	0.20	**L-Lysine**	8	2.45	0.39	**L-Cystine**	10	1.72	0.30
Lactose	10	1.71	0.53	**Glycerophospho-choline**	10	2.38	0.47	**Isocitric Acid**	9	1.65	0.82

L-Cystine	10	1.64	0.30
L-Phenylalanine	10	1.62	0.63
Isocitric Acid	10	1.55	0.55
2-Aminoadipic Acid	10	1.51	0.33
G-6-P	10	1.49	0.40
2-Deoxycytidine	10	1.49	0.42
Creatine	5	1.39	2.15
L-Anserine	8	1.32	0.93
Glyceric Acid	7	1.24	0.97
Pyroglutamic Acid	10	1.22	0.26
Ascorbic Acid	9	1.19	0.60
Hypotaurine	10	1.16	0.34
Trans-Acotinic Acid	10	1.12	0.47
HippuricAcid	10	1.12	0.31
3-Phenylpropionic Acid	10	1.08	0.33
Glutaconic Acid	10	1.03	0.13
UMP	10	1.02	0.13
L-Asparagine	7	1.01	0.74
L-Histidine	10	1.01	0.28
L-Carnosine	9	0.98	0.43
Adipic Acid	10	0.97	0.20
dAMP	10	0.96	0.44
D-Mannose	7	0.95	0.73
trans-4-Hydroxy-L-Proline	8	0.95	0.56
D-Fucose	10	0.94	0.17
5-Aminovaleric Acid	10	0.94	0.22
D-Fructose	8	0.90	0.54
Dimethylglycine	9	0.85	0.97
CMP	9	0.84	0.35
N-Acetylglycine	10	0.84	0.27
IMP	9	0.81	0.48
4-Hydroxyphenyl Acetic Acid	9	0.79	0.39
Spermidine	10	0.76	0.15
L-Tyrosine	10	0.74	0.40
Methylmalonic Acid	10	0.73	0.20
Dimethylamine	10	0.72	0.12
GMP	8	0.70	0.55
Pimelic Acid	10	0.66	0.10

Phenethylamine	10	2.22	0.87
L-Cystine	10	2.04	0.47
Trans-Acotinic Acid	10	2.00	0.90
Glyceric Acid	10	1.96	0.58
L-Phenylalanine	10	1.95	1.00
Glycogen	10	1.88	0.30
G-6-P	9	1.80	0.67
2-Aminoadipic Acid	10	1.80	0.52
Isocitric Acid	9	1.73	0.84
Pyroglutamic Acid	10	1.68	0.32
L-Anserine	10	1.64	0.31
Lactose	10	1.56	0.46
Ascorbic Acid	10	1.54	0.61
L-Carnosine	10	1.51	0.30
Hypotaurine	10	1.46	0.36
Ethanolamine	7	1.38	1.08
trans-4-Hydroxy-L-Proline	10	1.36	0.32
D-Mannose	9	1.34	0.60
Hippuric Acid	10	1.28	0.31
D-Galactose	5	1.24	1.45
3-Phenylpropionic Acid	10	1.20	0.37
2-Deoxycytidine	9	1.19	0.72
4-Hydroxyphenyl Acetic Acid	10	1.16	0.52
Dimethylamine	10	1.07	0.18
dAMP	10	1.06	0.61
UMP	10	1.05	0.21
Glutaconic Acid	10	1.05	0.36
Dimethyl sulphone	10	1.02	0.25
5-Aminovaleric Acid	10	0.95	0.16
Adipic Acid	10	0.94	0.20
L-Histidine	9	0.90	0.39
L-Methionine	9	0.88	0.54
Methylguanidine	10	0.87	0.40
Indoxylsulfate	9	0.84	0.55
D-Fucose	10	0.83	0.21
L-Tyrosine	9	0.77	0.42
N-Acetylglycine	8	0.73	0.43
Spermidine	9	0.73	0.32

Pyroglutamic Acid	10	1.55	0.34
2-Aminoadipic Acid	10	1.48	0.21
D-Galactose	6	1.41	1.27
L-Phenylalanine	10	1.40	0.31
D-Fructose	10	1.33	0.54
Trans-Acotinic Acid	10	1.31	0.51
Glyceric Acid	8	1.28	0.82
Hypotaurine	10	1.27	0.28
D-Gluconic Acid	4	1.26	1.63
L-Histidine	10	1.25	0.61
G-6-P	8	1.22	0.73
Hippuric Acid	9	1.19	0.52
Ascorbic Acid	10	1.18	0.46
L-Anserine	10	1.18	0.49
L-Carnosine	10	1.16	0.27
Glutaconic Acid	10	1.14	0.11
UMP	10	1.14	0.26
D-Mannose	8	1.08	0.68
2-Deoxycytidine	8	1.06	0.64
3-PhenylPropionic Acid	10	1.02	0.13
L-Asparagine	6	0.97	0.92
5-Aminovaleric Acid	10	0.97	0.19
CMP	9	0.96	0.38
4-Hydroxyphenyl Acetic Acid	10	0.95	0.44
Ethanolamine	5	0.94	1.04
trans-4-Hydroxy-L-Proline	8	0.94	0.58
Dimethylamine	10	0.93	0.14
Adipic Acid	10	0.91	0.19
dAMP	9	0.85	0.56
Dimethylglycine	6	0.84	1.24
D-Fucose	10	0.77	0.19
N-(2-Furoyl)Glycine	5	0.76	0.93
Dimethyl sulphone	10	0.75	0.12
L-Methionine	8	0.73	0.48
L-Tyrosine	10	0.70	0.36
L-Aspartate	6	0.70	0.66
1-Methylhydantoin	6	0.69	0.73
L-Glutathione-oxidized	9	0.64	0.35

L-Methionine	7	0.60	0.47	Betaine	9	0.73	0.60	IMP	7	0.64	0.52
L-Aspartate	5	0.59	0.64	Acetaminophen	7	0.68	0.53	Betaine	9	0.64	0.29
Valerate	10	0.59	0.12	IMP	8	0.68	0.62	GTP	8	0.63	0.34
Levoglucosan	7	0.58	0.42	Levoglucosan	7	0.67	0.48	Pimelic Acid	10	0.63	0.09
L-Alanine	9	0.57	0.34	Acetoacetate	10	0.65	0.32	N-Acetylglycine	8	0.61	0.44
Dimethyl sulphone	10	0.56	0.10	GTP	8	0.64	0.37	Spermidine	8	0.61	0.37
L-Isoleucine	10	0.56	0.09	L-Glutathione-oxidized	8	0.64	0.48	L-Isoleucine	10	0.59	0.09
Isovaleric Acid	10	0.54	0.10	Methylmalonic Acid	10	0.60	0.15	Valerate	10	0.55	0.09
Indoxylsulfate	7	0.53	0.40	3-Methylxanthine	9	0.60	0.74	Isovaleric Acid	10	0.54	0.11
3-Methylxanthine	7	0.53	0.90	L-Isoleucine	10	0.59	0.12	4-Aminohippuric Acid	8	0.54	0.66
L-Glutathione-oxidized	7	0.51	0.37	Pimelic Acid	10	0.58	0.09	Methylmalonic Acid	10	0.54	0.16
3-Hydroxybutyrate	10	0.50	0.04	L-Alanine	9	0.55	0.28	Dihydrothymine	9	0.50	0.35
4-Ethylphenol	8	0.48	0.27	L-Aspartate	4	0.54	0.71	3-Hydroxybutyrate	9	0.49	0.19
Myo-Inositol	7	0.47	0.37	Homovanillic Acid	7	0.54	0.50	Creatine	4	0.48	0.62
GTP	6	0.46	0.41	Valerate	10	0.53	0.07	L-Alanine	9	0.46	0.20
Betaine	8	0.45	0.28	CMP	6	0.52	0.47	Indoxylsulfate	6	0.45	0.42
1-Methylhydantoin	3	0.41	0.74	GMP	6	0.52	0.64	trans-Ferulic Acid	10	0.44	0.13
N-Acetyl-L-Aspartic Acid	10	0.41	0.06	Quinolinic Acid	5	0.52	0.57	UDPG	7	0.44	0.42
Quinolinic Acid	4	0.40	0.52	3-Hydroxybutyrate	10	0.52	0.12	L-Leucine	10	0.44	0.07
L-Leucine	10	0.40	0.04	Benzoic Acid	7	0.50	0.40	Acetoacetate	10	0.43	0.19
2-Oxobutyrate	10	0.40	0.05	trans-Ferulic Acid	10	0.50	0.12	N-Acetyl-L-Aspartic Acid	10	0.42	0.05
Acetaminophen	5	0.39	0.50	O-Acetyl-L-Carnitine	10	0.49	0.15	O-Acetyl-L-Carnitine	10	0.38	0.12
Benzoic Acid	6	0.38	0.35	4-EthylPhenol	9	0.48	0.22	Methylguanidine	10	0.38	0.05
trans-Ferulic Acid	9	0.37	0.15	Isovaleric Acid	10	0.47	0.13	Quinolinic Acid	4	0.38	0.49
Methylguanidine	10	0.35	0.06	N-Acetyl-L-Aspartic Acid	10	0.47	0.13	4-EthylPhenol	8	0.37	0.23
O-Acetyl-L-Carnitine	10	0.35	0.18	3-Hydroxyphenyl Acetic Acid	9	0.42	0.17	Benzoic Acid	6	0.37	0.33
N-(2-Furoyl)Glycine	2	0.35	0.89	2-Oxobutyrate	10	0.37	0.02	Levoglucosan	5	0.34	0.37
Acetoacetate	10	0.33	0.16	D-Fructose	3	0.36	0.62	Acetaminophen	4	0.34	0.46
4-Aminohippuric Acid	7	0.30	0.21	L-Leucine	10	0.36	0.06	2-Oxobutyrate	9	0.34	0.12
Pantothenic Acid	8	0.29	0.17	UDPG	5	0.32	0.41	Pyruvic Acid	10	0.31	0.06
UDPG	6	0.28	0.25	Pyruvic-Acid	10	0.32	0.05	3-Methylxanthine	9	0.31	0.18
Homovanillic Acid	5	0.27	0.29	Propylene Glycol	8	0.27	0.15	Homovanillic Acid	7	0.31	0.28
Ethanolamine	1	0.26	0.82	Trimethylamine	10	0.26	0.12	2-Hydroxyphenyl Acetic Acid	8	0.30	0.18
Propylene Glycol	7	0.26	0.21	4-Aminohippuric Acid	5	0.25	0.26	Propylene Glycol	9	0.28	0.11
Pyruvic-Acid	9	0.26	0.10	Myo-Inositol	3	0.25	0.41	Pantothenic Acid	7	0.24	0.18
3-Hydroxyphenyl Acetic Acid	6	0.23	0.21	Dihydrothymine	6	0.23	0.20	GMP	4	0.24	0.34
2-Hydroxyphenyl Acetic Acid	5	0.21	0.23	L-Valine	7	0.17	0.12	Myo-Inositol	3	0.23	0.37
Lactate	5	0.20	0.22	Lactate	4	0.15	0.20	3-Hydroxyphenyl Acetic Acid	6	0.22	0.20
Sebacic Acid	5	0.19	0.21	Pantothenic Acid	4	0.15	0.19	Lactate	6	0.22	0.19

Dihydrothymine	5	0.17	0.18	**β-Hydroxy-isovaleric Acid**	10	0.13	0.01	**β-Hydroxy-isovaleric Acid**	10	0.15	0.06
β-Hydroxy-isovaleric Acid	8	0.11	0.06	**2-Hydroxyphenyl Acetic Acid**	3	0.11	0.19	**Trimethylamine**	10	0.13	0.06
Trimethylamine	8	0.10	0.08	**N-(2-Furoyl)Glycine**	1	0.10	0.31	**Sebacic Acid**	2	0.09	0.21
L-Valine	2	0.05	0.11	**Sebacic Acid**	1	0.03	0.11	**L-Valine**	1	0.02	0.08

Data are expressed as mean and SD. n indicates the number of samples where the metabolite was detected. Relative concentration is an arbitrary unit. Refer to Table S1 for full names of metabolites.

Table S3. The (A) PCA loadings, (B) PLS-DA VIP scores and (C) PLS-DA diet regression coefficients for the top 20 urinary metabolites in 10 captive cheetahs. *All these metabolites contributed towards Table S4.*

A. PCA Metabolite loadings				**B. PLS-DA Metabolite VIP Scores**			
PC 1		**PC 2**		**C 1**		**C 2**	
Pantothenic acid	-0.173	UDPG	-0.220	Dihydrothymine	2.632	Dihydrothymine	2.185
L-Carnitine	0.173	Glutaconic acid	-0.210	Methylmalonic acid	2.404	Methylmalonic acid	1.794
Malonate	0.168	Trimethylamine	0.195	Dimethylamine	2.354	Dimethylamine	1.752
Choline chloride	0.162	D-Fructose	-0.192	Pyroglutamic acid	2.125	Pyroglutamic acid	1.647
Acetoacetate	0.161	Spermidine	0.189	L-Proline	2.024	GMP	1.594
L-Proline	0.160	Dimethylglycine	0.188	GMP	1.941	N-(2-Furoyl)Glycine	1.565
Isovaleric acid	-0.156	Methylguanidine	0.178	1,3-Diaminopropane	1.929	L-Proline	1.552
Acetaminophen	0.155	Lactate	-0.178	D-Fucose	1.894	Adipic acid	1.521
Taurine	0.154	L-Histidine	-0.177	Dimethyl sulphone	1.696	1,3-Diaminopropane	1.467
Ethanolamine	0.153	2-Deoxycytidine	0.174	L-Glutamine	1.610	UMP	1.454
Acetyl-L-carnitine	0.148	N-Acetylglycine	0.169	2-Deoxycytidine	1.557	Acetaminophen	1.446
Quinolinic acid	0.148	Trans-4-Hydroxy-L-Proline	0.168	D-Fructose	1.482	Dimethyl sulphone	1.416
Pyroglutamic acid	0.144	Threonic acid	-0.167	Ethanolamine	1.427	D-Fucose	1.411
Adipic acid	0.142	L-Glutamine	-0.160	Taurine	1.426	L-Alanine	1.380
L-Cystine	0.141	1-Methylhydantoin	0.151	Myo-Inositol	1.390	Lactose	1.337
L-Asparagine	0.140	Levoglucosan	0.146	TMAO	1.370	Isovaleric acid	1.335
Lactose	-0.140	Phenethylamine	0.146	D-Galactose	1.367	L-Glutamine	1.302
TMAO	0.139	Dihydrothymine	-0.141	N-Acetylglycine	1.330	Pimelic acid	1.255
Glycerophospho-choline	0.139	Dimethyl sulphone	0.140	UMP	1.329	L-Cystine	1.250
D-Galactose	-0.139	D-Gluconic acid	0.134	Levoglucosan	1.315	D-Galactose	1.240

C. PLS-DA metabolite coefficients for each diet					
Baseline		**Control**		**Glycine**	
Dihydrothymine	97.8	Methylguanidine	71.91	Dihydrothymine	100.0
Dimethyl sulphone	88.1	Dimethyl sulphone	68.06	Dimethyl sulphone	79.8
Dimethylamine	84.4	Trimethylamine	60.96	N-(2-Furoyl)Glycine	75.8
N-(2-Furoyl)Glycine	71.7	Dimethylglycine	56.15	GMP	74.1
1,3-Diaminopropane	71.7	2-Oxoglutarate	52.12	Pimelic acid	68.3

Methylmalonic acid	71.2	D-Fructose	50.13	Dimethylamine	68.0
GMP	70.9	1-Methylhydantoin	49.08	Methylmalonic acid	64.8
Pimelic acid	63.7	D-Glucose	46.90	1,3-Diaminopropane	60.6
L-Proline	61.5	Dimethylamine	46.77	Adipic acid	59.4
UMP	60.4	L-Valine	44.56	L-Tryptophane	54.8
Adipic acid	57.1	L-Carnosine	44.30	4-Aminohippuric acid	54.2
Pyroglutamic acid	54.9	3-Hydroxyphenylacetic acid	42.61	CMP	53.5
L-Alanine	54.2	L-Asparagine	40.27	UMP	51.3
CMP	52.4	Pimelic acid	38.89	L-Valine	50.6
D-Fructose	51.6	D-Maltose	38.31	L-Proline	50.6
4-Aminohippuric acid	49.4	Beta-Alanine	37.77	D-Glucose	48.3
Acetaminophen	48.7	Trans-Acotinic acid	37.32	Trimethylamine	48.2
L-Valine	47.3	L-Proline	36.98	2-Hydroxyphenylacetic acid	48.0
Trimethylamine	47.1	D-Glucose-6-Phosphate	33.69	Acetaminophen	47.8
D-Fucose	46.3	Pyroglutamic acid	32.96	D-Fructose	47.6

Loadings are the weight of each metabolite and are expressed as positive or negative number depending on its direction from the PC. Coefficients are the weighted sum of the PLS-DA regression for each group. PCA, principle component analysis; PLS-DA, partial least squares discriminant analysis, VIP, variable importance in projection; PC, principle component; C, component. Refer to Table S1 for full names of metabolites.

Table S4. The list of 65 potentially important urinary metabolites according to their frequency in the top 20% in univariate and multivariate analyses.

Metabolite	Count	Univariate	PC 1	PC 2	VIP 1	VIP 2	Coeff. Baseline	Coeff. Control	Coeff. Glycine
Dimethyl sulphone	**7**	1		1	1	1	1	1	1
L-Proline	**7**	1	1		1	1	1	1	1
D-Fructose	**6**	1		1	1		1	1	1
Dimethylamine	**6**	1			1	1	1	1	1
Pyroglutamic Acid	**6**	1	1		1	1	1	1	
1,3-Diaminopropane	**5**	1			1	1	1		1
Dihydrothymine	**5**			1	1	1	1		1
Methylmalonic Acid	**5**	1			1	1	1		1
Pimelic Acid	**5**	1				1	1	1	1
Trimethylamine	**5**	1		1			1	1	1
Acetaminophen	**4**		1			1	1		1
Adipic Acid	**4**		1			1	1		1
GMP	**4**				1	1	1		1
L-Valine	**4**	1					1	1	1
UMP	**4**				1	1	1		1
1-Methylhydantoin	**3**	1		1				1	
CMP	**3**	1					1		1
D-Fucose	**3**				1	1	1		
D-Galactose	**3**		1		1	1			
D-Glucose	**3**	1						1	1
Dimethylglycine	**3**	1		1				1	
Isovaleric Acid	**3**	1	1			1			
L-Asparagine	**3**	1	1					1	

L-Cystine	**3**	1	1			1			
L-Glutamine	**3**			1	1	1			
Methylguanidine	**3**	1		1				1	
N-(2-Furoyl)Glycine	**3**					1	1		1
Taurine	**3**	1	1		1				
TMAO	**3**	1	1		1				
2-Deoxycytidine	**2**			1	1				
2-Oxoglutarate	**2**	1						1	
3-Hydroxyphenyl Acetic Acid	**2**	1						1	
4-Aminohippuric Acid	**2**						1		1
Acetoacetate	**2**	1	1						
β-Alanine	**2**	1						1	
D-Maltose	**2**	1						1	
Ethanolamine	**2**		1		1				
Lactose	**2**		1			1			
L-Alanine	**2**					1	1		
L-Carnitine	**2**	1	1						
L-Carnosine	**2**	1						1	
Levoglucosan	**2**			1	1				
Malonate	**2**	1	1						
N-Acetylglycine	**2**			1	1				
Trans-Acotinic Acid	**2**	1						1	
2-Hydroxyphenyl Acetic Acid	**1**								1
Choline Chloride	**1**		1						
D-Gluconic Acid	**1**			1					
D-Glucose-6-Phosphate	**1**							1	
Glutaconic Acid	**1**			1					
Glycerophosphocholine	**1**		1						
Hypotaurine	**1**	1							
Lactate	**1**			1					
L-Histidine	**1**			1					
L-Tryptophane	**1**								1
Myo-Inositol	**1**				1				
O-Acetyl-L-Carnitine	**1**		1						
Pantothenic Acid	**1**		1						
Phenethylamine	**1**			1					
Phosphocholine	**1**	1							
Quinolinic Acid	**1**		1						
Spermidine	**1**			1					
Threonic Acid	**1**			1					
trans-4-Hydroxy-L-Proline	**1**			1					
UDPG	**1**			1					

Refer to Table S1 for full names of metabolites.

Table S5. Relative concentration of the 48 metabolites identified in the serum of captive cheetahs at baseline, on a control and glycine supplemented diet (n=10).

Baseline Diet				Control Diet				Glycine Diet			
Metabolite	**n**	**Mean**	**SD**	**Metabolite**	**n**	**Mean**	**SD**	**Metabolite**	**n**	**Mean**	**SD**
D-Glucose	10	23.96	2.97	D-Glucose	10	25.02	5.38	D-Glucose	10	23.41	3.01
L-Glutamine	10	4.89	0.60	L-Glutamine	10	4.93	0.51	L-Glutamine	10	4.68	0.40
Lactate	10	3.54	0.63	Lactate	10	3.66	0.43	Lactate	10	3.78	0.49
Taurine	10	2.74	1.02	Taurine	10	3.50	0.97	Taurine	10	2.89	0.87
Guanidinoacetic Acid	10	2.72	0.61	Guanidinoacetic Acid	10	2.90	0.79	Guanidinoacetic Acid	10	2.61	0.47
D-Mannose	10	2.58	0.33	D-Mannose	10	2.43	0.31	G-6-P	10	2.46	0.18
G-6-P	10	2.48	0.26	D-Glucuronic Acid	10	2.38	0.48	D-Mannose	10	2.37	0.34
Glyceric Acid	10	2.33	0.36	G-6-P	10	2.31	0.18	D-Glucuronic Acid	10	2.21	0.41
D-Glucuronic Acid	10	2.27	0.36	Glyceric Acid	9	2.28	0.40	Glyceric Acid	10	2.16	0.42
D-Galactose	9	1.96	0.27	D-Galactose	7	1.98	0.33	D-Galactose	10	1.79	0.28
L-Carnitine	10	1.76	0.43	L-Alanine	10	1.74	0.22	L-Alanine	10	1.74	0.26
L-Alanine	10	1.74	0.23	L-Carnitine	10	1.69	0.59	L-Carnitine	10	1.64	0.46
D-Maltose	10	1.61	0.17	D-Maltose	9	1.55	0.19	D-Maltose	10	1.58	0.12
L-Glycine	10	1.51	0.31	L-Glycine	10	1.48	0.47	L-Cystine	10	1.48	0.17
L-Cystine	10	1.50	0.14	L-Cystine	10	1.47	0.22	L-Glycine	10	1.40	0.32
D-Fructose	7	1.23	0.42	D-Fructose	7	1.47	0.39	L-Proline	10	1.25	0.19
L-Proline	10	1.20	0.16	Ethanolamine	10	1.24	0.27	Ethanolamine	10	1.19	0.21
Ethanolamine	10	1.14	0.14	L-Proline	10	1.24	0.29	Creatinine	10	1.10	0.18
Creatinine	10	1.10	0.19	L-Lysine	6	1.12	0.22	D-Sorbitol	8	1.08	0.17
D-Sorbitol	9	1.02	0.20	Creatinine	10	1.09	0.13	D-Fructose	6	1.07	0.41
Galactitol	10	0.98	0.19	D-Sorbitol	10	1.05	0.23	Galactitol	10	0.96	0.17
Betaine	9	0.93	0.21	Galactitol	10	1.03	0.22	Betaine	10	0.94	0.27
L-Glutamic Acid	9	0.89	0.10	Betaine	8	0.99	0.32	Phosphocholine	8	0.91	0.11
Threitol	10	0.88	0.11	Phosphocholine	9	0.95	0.22	Ascorbic Acid	10	0.87	0.14
Ascorbic Acid	10	0.87	0.11	Ascorbic Acid	10	0.93	0.18	L-Lysine	7	0.87	0.19
Phosphocholine	10	0.87	0.20	Threitol	9	0.91	0.16	Threitol	9	0.86	0.14
α-Aminobutyric Acid	10	0.87	0.10	α-Aminobutyric Acid	10	0.78	0.10	α-Aminobutyric Acid	10	0.78	0.11
D-Gluconic Acid	8	0.75	0.11	D-Gluconic Acid	8	0.76	0.13	Lactose	10	0.78	0.14
L-Leucine	10	0.72	0.17	Lactose	10	0.72	0.11	L-Leucine	10	0.68	0.07
Lactose	10	0.69	0.11	L-Leucine	10	0.67	0.06	L-Threonine	10	0.59	0.11
Hypotaurine	10	0.57	0.03	Hypotaurine	10	0.57	0.05	Hypotaurine	10	0.55	0.05
Citrate	10	0.54	0.11	N-Acetylglycine	10	0.50	0.04	D-Gluconic Acid	7	0.53	0.38
N-Acetylglycine	10	0.49	0.04	L-Threonine	8	0.44	0.25	N-Acetylglycine	10	0.48	0.03
Cadaverine	9	0.48	0.18	Pantothenic Acid	10	0.43	0.03	Citrate	8	0.46	0.26
Pantothenic Acid	10	0.45	0.03	Choline Chloride	10	0.42	0.21	Choline Chloride	10	0.45	0.28
L-Threonine	8	0.44	0.26	Citrate	8	0.42	0.23	Pantothenic Acid	10	0.43	0.03
Propylene Glycol	10	0.41	0.04	Propylene Glycol	10	0.40	0.07	Propylene Glycol	10	0.41	0.04

L-Valine	10	0.39	0.13	L-Valine	10	0.36	0.06	L-Valine	10	0.40	0.07
L-Lysine	4	0.37	0.50	Cadaverine	6	0.32	0.29	Cadaverine	8	0.40	0.22
Propionate	8	0.36	0.19	Creatine	7	0.32	0.23	L-Tyrosine	10	0.31	0.03
Choline Chloride	10	0.29	0.05	L-Tyrosine	9	0.28	0.10	L-Methionine	5	0.26	0.28
Phenethylamine	6	0.26	0.23	L-Methionine	5	0.27	0.29	Creatine	5	0.26	0.31
L-Tyrosine	8	0.25	0.13	1,3-DAP	5	0.26	0.28	Phenethylamine	6	0.26	0.22
Creatine	4	0.24	0.37	L-Glutamic Acid	3	0.26	0.42	L-Glutamic Acid	2	0.22	0.46
α-Hydroxyisobutyric Acid	9	0.21	0.11	Propionate	6	0.25	0.21	1,3-DAP	4	0.20	0.26
1,3-DAP	3	0.14	0.23	α-Hydroxy-isobutyric Acid	9	0.24	0.11	α-Hydroxy-isobutyric Acid	9	0.20	0.09
L-Methionine	2	0.10	0.21	Phenethylamine	5	0.21	0.22	Propionate	3	0.13	0.21
O-Acetyl-L-Carnitine	9	0.08	0.03	O-Acetyl-L-Carnitine	9	0.08	0.03	O-Acetyl-L-Carnitine	9	0.08	0.03

Data are expressed as mean and SD. n indicates the number of samples where the metabolite was detected. Relative concentration is an arbitrary unit. Refer to Table S1 for full names of metabolites.

Table S6. The list of 32 potentially important serum metabolites according to their frequency in the top 20% in univariate and multivariate analyses.

Metabolite	Count	Univariate	PC 1	PC 2	VIP 1	VIP 2	Coeff. Baseline	Coeff. Control	Coeff. Glycine
L-Glutamic acid	6	1			1	1	1	1	1
L-Threonine	5			1	1	1		1	1
α-Aminobutyric Acid	4	1			1	1	1		
D-Glucose-6-Phosphate	4					1	1	1	1
Ethanolamine	4		1			1		1	1
L-Methionine	4				1	1	1		1
Propionate	4				1	1	1		1
Choline Chloride	3				1	1	1		
D-Mannose	3				1	1	1		
L-Lysine	3			1	1		1		
L-Valine	3			1				1	1
Taurine	3						1	1	1
Cadaverine	2			1			1		
D-Galactose	2							1	1
D-GlucuronicAcid	2		1					1	
D-Maltose	2							1	1
Lactate	2			1	1				
Lactose	2				1	1			
Ascorbic Acid	1							1	
Creatine	1			1					
Creatinine	1			1					
D-Gluconic Acid	1			1					
D-Glucose	1		1						
Guanidinoacetic Acid	1		1						
Hypotaurine	1		1						
L-Carnitine	1		1						
L-Cystine	1		1						
L-Glutamine	1			1					

L-Glycine	1	1	
N-Acetylglycine	1	1	
Phenethylamine	1		1
Propylene Glycol	1	1	

Refer to Table S1 for full names of metabolites.

Table S7. Description of cheetahs included in the study (n=10)

Group	Number	Sex	Age (y)	Siblings
2	1	Male	5	*
	2	Male	5	*
	3	Male	5	*
	4	Female	3	#
1	5	Female	5	*
	6	Male	4	No
	7	Female	3	#
	8	Female	3	#
	9	Female	2	+
	10	Male	2	+

Age is the age of the individual at the end of the sample collection period. Matching symbols (, #, +) indicate individuals that are siblings.*

Supplementary Figures

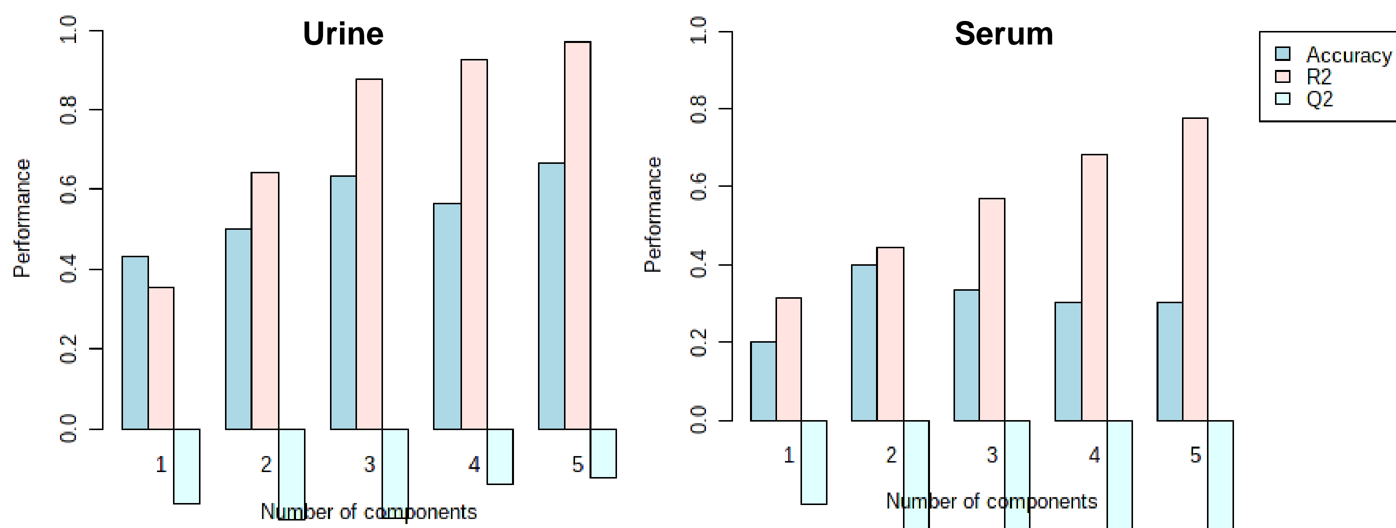

Figure S1. PLS-DA classification for urine (left) and serum (urine) of captive cheetahs using different numbers of components where the leave-one-out validation was used. *None of the components represent a good classifier. Accuracy, predicted residual error sum of squares (PRESS); R2, sum of squares; Q2, cross-validated R2.*

APPENDIX 2

Statistical analysis of the urine and serum metabolites of the n=6 group: Methods and Results

The following analyses were conducted:

- The identification and quantification of the metabolites present in the urine and serum of 6 (Group 1) captive cheetahs at baseline, on a control diet and on a glycine supplemented diet.

- The same process was followed as described in Chapter 2 *"Data analysis and Statistical analysis"*. The most notable difference in this process was the data cleaning and normalisation (auto scaling) which was performed in MetaboAnalyst 5.0. Data cleaning removes metabolites that are present in less than 50% of all three groups in order to reduce the number of outlying metabolites that may affect the analysis. Auto scaling makes use of the following equation to calculate the scaled value for a single metabolite of a sample: scaled value = $(X_2 - X_1)/SD_1$ where X_2 is an individual sample metabolite concentration, X_1 is the metabolite mean for all samples, SD_1 is the metabolite standard deviation for all samples. As the sample size was different to the n=10 analysis, this would lead to different metabolite means and standard deviations, resulting in different scaled values which would then undergo the respective univariate and multivariate statistical analyses.
- Univariate (one way repeated measures ANOVA) and multivariate (PCA and PLS-DA) analyses were performed and the metabolites of interest were identified as described in Chapter 2 *"Identifying metabolites of interest".*
- The urine and serum PCA and PLS-DA plots were compared between the combined n=10 group (from Chapters 4 and 5) and the split n=6 group. The identified metabolites of interest were evaluated to determine whether this process would lead to different overall conclusions.

The results of the additional analyses are described below.

Urine metabolites

After cleaning, there were 117 identified urinary metabolites remaining compared to the 112 metabolites in the n=10 analysis. MetaboAnalyst identified 10 significant metabolites with a one-way repeated measures ANOVA after FDR adjustment, compared to the 31 significant metabolites identified previously. These 10 metabolites were dimethyl sulphone, α-ketoglutaric acid, methylguanidine, L-leucine, trimethylamine, dimethylamine, dimethylglycine, D-fructose, pimelic acid and TMAO (Table S8). L-leucine was the only metabolite not previously identified in the n=10 group analysis.

Table S8. Significant metabolites identified by univariate analyses in the urine of captive cheetahs at baseline, on a control diet and glycine supplemented diet (n=6)

Metabolite	F-value	Adjusted p-value	Tukey-Kramer p-value		
			Baseline-Control	**Baseline-Glycine**	**Control-Glycine**
Dimethyl sulphone	72.78	0.0001	**<0.001** (C>B)	NS	**<0.001** (C>G)
α-ketoglutaric acid	54.72	0.0002	**<0.01** (C>B)	NS	**<0.05** (C>G)
Methylguanidine	38.84	0.0007	**<0.001** (C>B)	NS	**<0.001** (C>G)
L-Leucine	33.52	0.0010	NS	**<0.05** (G>B)	**<0.001** (G>C)
Trimethylamine	17.91	0.0110	**<0.001** (C>B)	NS	**<0.01** (C>G)
Dimethylamine	14.09	0.0159	**0.02** (C>B)	NS	NS
Dimethylglycine	13.53	0.0159	**<0.01** (C>B)	NS	**<0.001** (C>G)
D-Fructose	11.20	0.0370	NS	NS	**<0.01** (G>C)
Pimelic acid	11.19	0.0370	NS	NS	NS
TMAO	9.96	0.0444	NS	NS	NS

NS, not significant; TMAO, Trimethylamine oxide.

The PCA and PLS-DA plots for the baseline, control and glycine supplemented diet of the n=6 and n=10 group are shown in Figure S2. For the n=6 group, PC 1 accounts for 29.9% and PC 2 accounts for 13.2% of the variation observed. This was similar to the combined n=10 group with 27.5% (PC 1) and 9.5% (PC 2) of the variation. For the PLS-DA of the n=6 group, component 1 and 2 explained 18.2% and 17.8% of the variation, respectively. It is apparent from Figure S2 (right panel) that the spread of scores for each diet are more compact for the n=6 group compared to the n=10 group and when viewed together may indicate a slightly better separation. Although the cross validation Q2 values were still negative (data not shown) which indicates that this still has poor predictive power. When separated by diet, there is clear overlap and similar pattern between the groups – with the control diet having the greatest spread of data. There were slightly different metabolites driving the variation in the n=6 group for the PCA and PLS-DA with the top three metabolite in each analyses as follows: pantothenic acid, choline chloride, L-carnitine (PC 1 loadings); L-leucine, dimethyl sulphone, methylguanidine (PC 2 loadings); ethanolamine, L-proline, dimethylamine (VIP 1); ethanolamine, allantoin, L-proline (VIP 2); ethanolamine, dimethylamine, allantoin (baseline coefficient); dimethyl sulphone, methylguanidine, dimethylglycine (control coefficient); dimethyl sulphone, L-leucine, dimethylamine (glycine coefficient). This slight difference in individual metabolites was expected because of the difference in scores due to the different normalisation values and sample size compared to the n=10 group.

Seven metabolites of interest were identified in the top 20% in 5 or more analyses – namely L-leucine, D-fructose, spermidine, dimethyl sulphone, dimethylamine, L- proline and guanosine monophosphate (GMP). Fructose, dimethyl sulphone, dimethylamine and L-proline were identified previously as metabolites of interest and clearly highlighted and discussed in Chapter 4 and 6. Spermidine was not previously highlighted to the same extent, although it was discussed in Chapter 4 and 6 in relation to

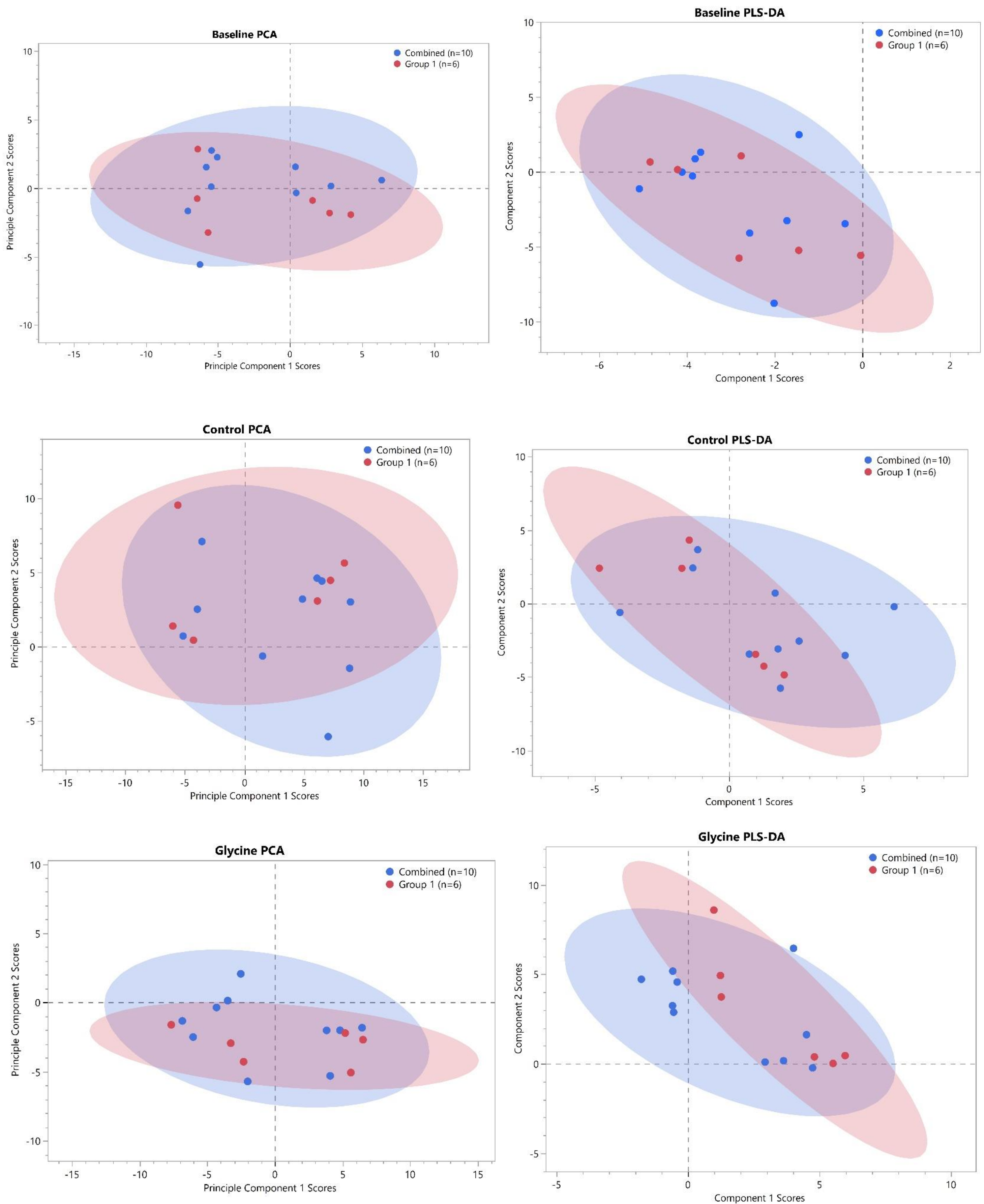

Figure S2. A two dimensional PCA (left) and PLS-DA (right) scores plot of the metabolites identified in the urine of captive cheetahs at baseline (top panel), on the control (middle panel) and glycine supplemented (bottom panel) diets. The combined n=10 group is reflected in blue and the split n=6 group is reflected in red. *Each point represents an individual cheetah with a corresponding 95% confidence ellipse for each group.*

1,3-diaminopropane as a polyamine in the "methionine associated pathway". The purine ribonucleotide, GMP, was previously identified in 4 urinary analyses (Table S4), but was not discussed. However, it may have a very close link to the pyrimidines, dihydrothymine and CMP, which were highlighted and discussed in Chapter 4 and 6. This would likely provide a further support for the role that glycine plays in purine synthesis and, therefore, DNA and RNA synthesis which was discussed in the aforementioned chapters. The essential amino acid L-leucine was not identified in any of the previous analyses, likely due to its fairly constant urinary concentration across the combined n=10 group (Table S2). However, in the n=6 group there was a significantly higher concentration of L-leucine in the glycine supplemented diet compared to the baseline and control diet. L-leucine is closely linked to protein synthesis (through activation of mTOR) and the activation of glutamate dehydrogenase which catalyses the inter-conversion of glutamic acid to α-ketoglutaric acid (Wu, 2009). Although it was not specifically discussed, it has a close connection to the discussion of glutamic acid as well as energy production through its role as a ketogenic amino acid contributing towards acetyl-CoA and acetoacetate.

Serum metabolites

After cleaning, there were 50 identified serum metabolites remaining compared to the 48 metabolites in the previous n=10 analysis. MetaboAnalyst did not identify any significant metabolites with a one-way repeated measures ANOVA after FDR adjustment, this was similar to the previous analysis. However, L-glutamine ($F = 10.09$; raw $p = 0.004$; adjusted $p = 0.188$), L-glutamic acid ($F = 7.26$; raw $p = 0.011$; adjusted $p = 0.259$), L-valine ($F = 6.02$; raw $p = 0.019$; adjusted $p = 0.259$), D-glucuronic acid ($F = 5.69$, raw $p = 0.022$; adjusted $p = 0.259$), L-carnitine ($F = 5.14$; raw $p = 0.029$; adjusted $p = 0.273$) and lactate ($F = 4.23$; raw $p = 0.047$; adjusted $p = 0.368$) were significant prior to FDR adjustment.

The serum PCA and PLS-DA plots of the combined n=10 and split n=6 group for each diet are shown in Figure S3. For the n=6 group, PC 1 accounts for 34.1% of the variation explained while PC 2 accounts for 13.1%. Similarly, in the n=10 group PC 1 accounted for 32.3% and PC 2 accounted for 13.1%. When the diet is considered through PLS-DA, component 1 accounts for 15.5% and component 2 accounts for 27.3% of the variation – this was similar to what was found in the previous analysis with clear overlap between diets. Therefore, this PLS-DA model did not have high predictive power for the different diets. There is clear overlap for the n=6 and n=10 group for each diet with potential outliers driving the spread of data despite the apparent clustering of the data. The top three metabolites driving the variation observed in multivariate analyses of the serum are as follows: guanidinoacetic acid, L-cystine, hypotaurine (PC 1 loadings); galactitol, choline chloride, D-sorbitol (PC 2 loadings); L-glutamic acid, galactitol, propionic acid (VIP 1); L-glutamic acid, galactitol, propionic acid (VIP 2); L-glutamic acid, galactitol, D-mannose (baseline coefficient); L-valine, L-glutamine, D-

glucose-6-phosphate (control coefficient); L-valine, L-glutamic acid, L-glutamine (glycine coefficient). The slight difference in metabolites would be the same as previously described for urine.

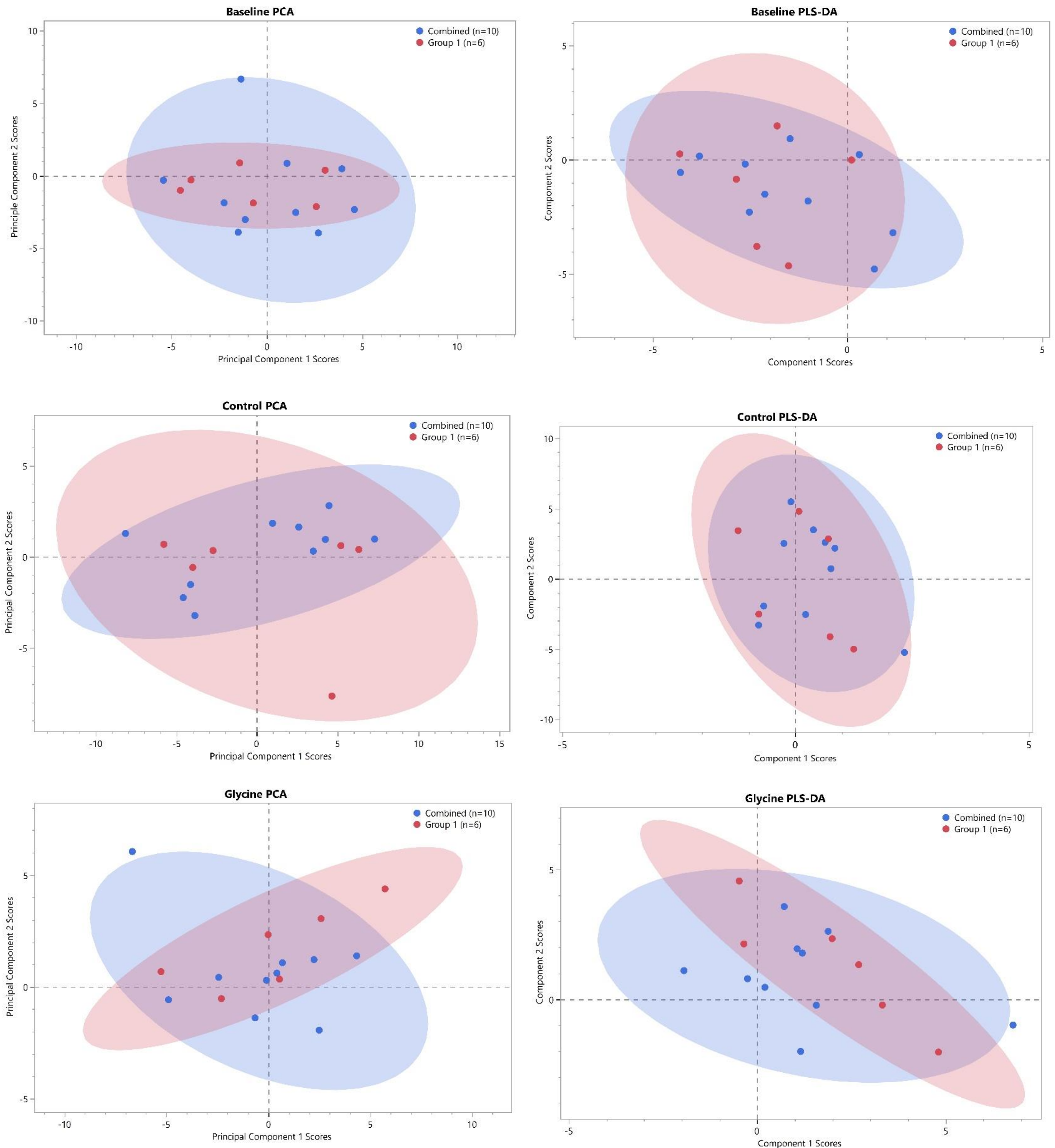

Figure S3. A two dimensional PCA (left) and PLS-DA (right) scores plot of the metabolites identified in the serum of captive cheetahs at baseline (top panel), on the control (middle panel) and glycine supplemented (bottom panel) diets. The combined n=10 group is reflected in blue and the split n=6 group is reflected in red. *Each point represents an individual cheetah with a corresponding 95% confidence ellipse for each group.*

Seven metabolites of interest were identified in the top 20% of 5 analyses – namely L-glutamic acid, D-glucuronic acid, D-sorbitol, galactitol, L-carnitine, L-threonine and L-valine. Glutamic acid and threonine were identified previously (to the same extent) and formed a crucial part of the discussion in Chapters 5 and 6. Indeed, their respective pathways were highlighted extensively under "*glutamic acid and threonine associated pathways*" and were influenced by glycine supplementation or deficiency. D-glucuronic acid was previously identified to a lesser extent, although it was briefly discussed in Chapter 6 in relation to glucose metabolism. The findings of D-sorbitol and galactitol are somewhat surprising in the cheetah with the former being discussed briefly in Chapter 4 in relation to fructose metabolism as it can be used to synthesis fructose through the polyol pathway in mammals. Both are sugar alcohols which can be reduced from glucose and galactose and, therefore, have a strong link to "*carbohydrate and fat metabolism*" which was extensively discussed in Chapter 6 despite these specific metabolites not being discussed. Carnitine was previously highlighted in the urine analysis and therefore discussed in Chapter 4 in relation to dimethylamine and trimethylamine. It was also discussed in Chapter 6 in relation to synthesis, transport and oxidation of fatty acids, and therefore under the broader pathway of "*carbohydrate and fat metabolism*". The essential amino acid, L-valine, was highlighted previously in the urine (Table 7, Table 8) and, to a lesser extent, in the serum (Table 10). It was discussed in Chapter 6 as a potential source of propionyl-CoA and under the larger pathway of "*threonine associated pathways*". Most of the serum metabolites of interest were linked to energy metabolism either through carbohydrate or fat metabolism which is notably less diverse than what was found in the n=10 analysis.

Summary and concluding thoughts on the combined n=10 and split n=6 group analysis

It is evident from the above that using a different grouping of the samples resulted in the identification of slightly different metabolites of interest. For the urine analysis, less metabolites were identified through univariate analysis, but these metabolites had a greater difference between diets i.e. a larger effect. For the serum analysis, more metabolites were identified by univariate analysis for the n=6 group, but none were significant after FDR adjustment. In the urine and serum PCA and PLS-DA, the two groups had a clear overlap indicating a similar metabolome albeit with slightly different scores (due to the different sample size) and metabolites driving the variation. Most of the key metabolites identified were found previously either to a similar or lesser extent. Therefore, the same or similar conclusions can be drawn in relation to specific pathways specifically that of glucose and fat metabolism, and purine and pyrimidine biosynthesis.

The combination of Group 1 (n=6) and Group 2 (n=4) is a clear study limitation as they experienced different lengths of time on the respective diets and when analysed separately produced slightly different results. However, utilising the combined n=10 group with a greater statistical power allowed the identification of a greater diversity of metabolites in the urine and the well-regulated and conserved

serum – providing a better representation of our untargeted approach. Therefore, we believe that utilising the combined n=10 group is a fair approach to investigate the holistic effect of glycine supplementation in captive cheetahs and address the aims of the study. It is able to provide us with more diverse information than using the n=6 group, which is notably less powered although it has the potential for a greater effect size.

UNIVERSITEIT VAN PRETORIA
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Faculty of Veterinary Science
Animal Ethics Committee

5 March 2020

Approval Certificate
New Application

AEC Reference No.: REC231-19
Title: The metabolic effects of glycine supplementation in captive cheetahs (Acinonyx jubatus).
Researcher: Ms KM Van Boom
Student's Supervisor: Prof ASW Tordiffe

Dear Ms KM Van Boom,

The **New Application** as supported by documents received between 2020-01-15 and 2020-02-24 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-02-24.
Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Cheetah	16
Samples Semen (depending on number of males)	24 (0.5-1ml)
Serum	48 (20 ml each)
Skeletal muscle (Triceps brachii)	48 (40-100mg)
Urine	48 (5 ml each)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-03-05.
3. Please remember to use your protocol number (REC231-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

11 May 2020

Approval Certificate
Amendment1

AEC Reference No.:	REC231-19
Title:	The metabolic effects of glycine supplementation in captive cheetahs (Acinonyx jubatus).
Researcher:	Ms KM Van Boom
Student's Supervisor:	Prof ASW Tordiffe

Dear Ms KM Van Boom,
The **Amendment** as supported by documents received between 2020-03-12 and 2020-05-04 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-05-04.

Please note the following about your ethics approval has been granted:

1. The second venue will be Cheetah Experience in Bloemfontein (1 Maluti Avenue, Bloemfontein, Free State).

   The study will be replicated at a) Cango Wildlife Ranch and b) Cheetah Experience.
   (The animal numbers will not change)

2. Please remember to use your protocol number (REC231-19) on any documents or correspondence with the AEC regarding your research.

3. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Faculty of Veterinary Science
Animal Ethics Committee

11 May 2020

Approval Certificate
Amendment 2

AEC Reference No.: REC231-19
Title: The metabolic effects of glycine supplementation in captive cheetahs (Acinonyx jubatus).
Researcher: Ms KM Van Boom
Student's Supervisor: Prof ASW Tordiffe

Dear Ms KM Van Boom,
The **Amendment** as supported by documents received between 2020-04-14 and 2020-05-04 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-05-04.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number Available**
Cheetah	
Samples Semen (depending on number of males)	24 (0.5-1ml)
Serum	48 (20 ml each)
Skeletal muscle (Triceps brachii)	48 (40-100mg)
Urine	48 (5 ml each)
Additional samples approved: Gastric mucosal biopsies (typically 2mm x 2mm x 2mm) will be collected from each animal This will be done by means of a 1.5m endoscope (Stortz, Germany).	9 per animal (three from each area in the stomach). Total 144

2. Please remember to use your protocol number (REC231-19) on any documents or correspondence with the AEC regarding your research.
3. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

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We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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