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

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Highlights

- Enteric bacterial phenolic compounds occur at high concentration in cheetah urine.
- Aromatic phenolic compounds are metabolised through glycine conjugation.
- A novel cadaverine metabolite N^1,N^5-dimethylpentane-1,5-diamine, was abundant.
- Pantothenic acid excretion correlated negatively with age.

Abstract

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

Keywords

Cheetahs, *Acinonyx jubatus*, urine organic acids, metabolomics, GC-MS.

Introduction

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

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

Urine is valuable from an analytical point of view as it contains a large number of metabolites, produced by homeostatic mechanism within an organism, that are likely to remain relatively unaltered if collected soon after capture and immobilization. Urinary organic acids are a broad class of water-soluble end-products and intermediates of a wide

range of metabolic pathways, including the metabolism of amino acids, carbohydrates, fatty acids, ketones and biogenic amines. The quantification of urinary organic acids has primarily been used in the detection and management of inborn errors of metabolism [14]. Changes in the urinary excretion of organic acids have, however, also provided valuable information for the early diagnosis of metabolic [15,16] and neurological disorders [17,18] and have broadened our understanding of the metabolic alterations associated with intestinal dysbiosis [19], and malnutrition in humans [20,21].

Despite their broad potential application [22,23], metabolome studies have, to date, rarely featured in veterinary studies. Comprehensive metabolic profiles of zoo or other captive wildlife species have not been reported in the literature and only a handful of metabolome studies have been conducted on free-ranging species [24-26]. Research on the urine metabolites in wild felids have been limited to the study of compounds that are considered important in territorial marking and other behaviours [27]. In the present study we document the organic acid metabolites in urine from cheetahs using gas chromatography-mass spectrometry (GC-MS) in order to develop a basic understanding of their metabolism, and generate new hypotheses for further investigations into the mechanisms of diseases they suffer from in captivity. The findings provide a baseline of reference values in cheetahs on which future research can be built.

Materials and Methods

1.1 Ethical and Permit considerations

The project was approved by the National Zoological Gardens of South Africa's Research and Ethics Committee (Project no. P11/07). A research/collecting permit (1846/2013) was

obtained from the Namibian Ministry of Environment and Tourism and the samples were imported into South Africa with the required CITES export (no.0042838) and import (no. 137670) permits, as well as a veterinary import permit (no. 13/1/1/30/2/10/6-2013/11/002397). Once in South Africa, the samples were transported and stored with the required national Threatened or Protected Species (TOPS) ordinary permit (no. 05238).

1.2 Sample collection

Urine was collected from 41 (23 males and 18 females) apparently healthy adult cheetahs, with ages ranging from 2 to 14 years housed at the AfriCat Carnivore Care Centre near Otjiwarongo in Namibia during routine annual health examinations. All of the cheetahs at the centre were wild-born and rescued as cubs (< 12 months of age) from commercial farmland in central Namibia. They either form part of AfriCat's rescue and release programme or are deemed unsuitable for release and are maintained in captivity for conservation education and research purposes. They are housed in large (> 1000 m^2) camps, covered by natural vegetation and are fed a diet consisting largely of donkey meat, supplemented with a combination of vitamins and minerals (FeliCal® and FeliVit®, Kyron Laboratories, South Africa).

The cheetahs were fasted for 12 to 24 hours and immobilized via remote injection dart (Daninject, Denmark) with medetomidine hydrochloride (Medetomidine 20mg/ml, Kyron Laboratories Pty LTD, South Africa) in combination with either zolazepam/teletamine (Zoletil®, Virbac, South Africa) or midazolam (Midazolam 50mg/ml, Kyron Laboratories Pty LTD, South Africa) and butorphanol (Butorphanol 50mg/ml, Kyron Laboratories Pty LTD, South Africa). Once sufficiently anesthetised, urine was collected within 15 minutes

into 5ml cryogenic vials (Corning, USA) after urethral catheterisation using a sterile 6FG dog urinary catheter (Buster Dog Catheter®, Kruuse, Denmark). The urine was centrifuged at 2000g for 5 minutes and the supernatant pipetted off into clean cryovials to ensure the exclusion of any cellular debris. The urine was then frozen immediately at -20°C and kept at this temperature until analysis.

1.3 Urine specific gravity and creatinine determination

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

1.4 Organic acid analysis

The methodology for the organic acid analysis was described by Rinaldo 2008 [28] and subsequently modified and performed as described by Reinecke *et al* [29]. In short, 100 µl of the internal standard (3-phenylbutyric acid, 52.5mg/dl, Sigma Chemical Company) was added to 1ml of urine and acidified to a pH < 2 with 5N HCl. The organic acids were extracted with 5 ml ethyl acetate and 3 ml diethyl ether consecutively by shaking for 15 minutes. After centrifugation, the organic phase was removed and pooled in a 10 ml Kimax tube dried with sodium sulphate, centrifuged again and transferred to a second clean Kimax tube, which was then dried under nitrogen. The dried organic acids were derivatized with O-bis(trimethylsilyl)trifluoroacetamide (BSTFA): trimethylchlorosilane (TMCS): pyridine (100:20:20 µl), for 45 minutes at 85 °C and 1 µl was injected into the GC-MS.

The Agilent GC–MS system used in this study consisted of a model 7890A gas chromatograph, a model 5975C mass selective detector, an HP 5970 C MS and Agilent Chemstation (Revision E.02.00). A fused-silica capillary column (DB-1MS UI, 30 m, 2.50 lm i.d., 0.25 lm film thickness) was used for the fractionation. The initial GC temperature was kept at 60°C 2 min. It was then increased to 120°C at a rate of 5°C /min, to 295°C at a rate of 7°C /min, and then held at a final temperature of 295 °C for 2 min. Helium (1 ml/min) was used as a carrier gas at a constant flow rate. The mass spectra of all GC peaks were generated by a mass spectrometer operated at 70 eV in the electron impact mode with SCAN (50–600 amu) positive ion monitoring. The MS source and quadrupole temperatures were 230 and 150°C respectively.

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

The Potchefstroom laboratory for Inborn Errors of Metabolism (PLIEM), where the analyses were done, participates in an external quality control program, Quality Assurance In Laboratory Testing For IEM (ERNDIMQA) http://www.erndimqa.nl/ . All quality control was done according to the specification of ERNDIMQA on material supplied by ERNDIMQA (Control Organic Acids, Product code- ORG-01, Batch number- 2016.007 (http://cms.erndimqa.nl/getdoc/440cb2de-9489-4e46-8477-84be0a9309bb/2016-007-PC-Org-

Acids_v1.aspx). However, due to the possible presence of hundreds of organic acids in urine and unavailability of standards for all the organic acids, a response factor of 1 was used to quantify organic acids that were not present in the ERNDIMQA sample material. The calculated concentrations (Calculated concentration = Area of metabolites/Area of internal standard x concentration of internal standard x response factor) were therefore relative to the response and concentration of the internal standard.

Metabolite concentrations were corrected relative to urine specific gravity (SG) as described by Miller *et al* [30], where:

Corrected concentration = Raw concentration x $(SG_{target} -1.0)/SG_{sample} - 1.0)$

The SG_{target} is a population mean SG, which in this study is equal to 1,054.

A data matrix was created by aligning all the metabolites against the samples using MATLAB [31].

1.5 Identification and synthesis of unknown metabolites

Tentative identification of metabolites was made by spectral comparison using the NIST Mass Spectral Search Program (Version 2.0.g). The definitive identification of unknown compounds was achieved by comparison to synthetically produced metabolite standards. N-acetylglycine conjugates were prepared using the method described by Furniss *et al* [32]. The acylchloride component of the organic acids (benzoate, phenylacetate, phenylpropionate, 4-hydroxyphenylacetate, 4-hydroxyphenylpriopionate and 4-hydroxyphenylacrylate) were

prepared as described by the same authors. The N-acetylglycine/glutamate conjugate was prepared by dissolving 0.5g of the amino acid in 10ml (10%) sodium bicarbonate to which 1 gram of acylchloride was added. The mixture was then mixed on a rotary wheel for an hour. The product was extracted with ethylactetate after lowering the pH to < 2 with 6 N HCl, dried, derivitized and then analysed using GC-MS.

1.6 Statistical analyses

All results are reported as mean concentrations and standard deviations. Spearman's rank correlation coefficients (r) and p-values were calculated for organic acid concentrations relative to age. Correlations were deemed important if $|r| \geq 0.3$ and $p \leq 0.05$. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed to compare the organic acid concentrations of male versus female cheetahs after auto scaling. PCA is an unsupervised (i.e. using no group information to explain observed variation) method used here to assess the generalizability of the supervised PLS-DA model. All analyses were done using IBM SPSS Statistics Version 22 [33] or MetaboAnalyst 3.0 (www.metaboanalyst.ca) software [34].

Results

A total of 339 different organic acids could be annotated and quantified from the GC-MS data. Of these metabolites 140 were not detectable in more than 80% of the samples and were therefore excluded, leaving 199 variables for statistical analysis. A representative chromatogram of the urinary organic acids of one of the cheetahs is shown in Figure 1. The mean concentrations of the thirty most abundant organic acids are listed in Table 1. The most

abundant organic acids in the cheetahs urine were an unidentified compound with a total molecular mass of 362 (annotated Unknown 362), and a compound tentatively identified as N^1,N^5-Dimethylpentane-1,5-diamine. The mass spectra of these two compounds are shown in Figures 2 and 3 respectively. The fragmentation of N^1,N^5-dimethylpentane-1,5-diamine is also shown in Figure 3. A number of glycine conjugates, were detected at relatively high concentrations. Krebs cycle intermediates, intermediate products of fatty acid metabolism and metabolites formed by the degradation of particularly the aromatic amino acids were also abundant.

Figure 1. Annotated chromatogram of the organic acids detected in the urine of a captive cheetah.

1. Lactic acid; 2. Oxalic acid; 3. Sulfate; 4. Urea; 5. Phosphate; 6. Succinic acid; 7. Unknown M^+=200; 8. 2,3-Dihydroxybutyric acid; 9. Glutaric acid; 10. Internal standard; 11. 3-Methylglutaconic acid; 12. N1,N5-Dimethylpentane-1,5-diamine; 13. Isovalerylglycine; 14. 5-Hydroxy hydantoin; 15. Unknown M^+=362; 16. Pimelic acid; 17. Hexanoylglycine; 18. 4-Hydroxyphenylacetic acid; 19. Suberic acid; 20. Aconitic acid; 21. 4-Hydroxymandelic acid; 22. Hippuric acid; 23.Isocitric acid; 24. Phenylacetylglycine; 25. Methylcitric acid; 26. Isovalerylglutamic acid; 27. Phenylpropionylglycine; 28. Pantothenic acid; 29. 4-Hydroxyphenylacetylglycine 30. 1H-Indole-3-acetic acid. 31. Benzoylglutamic acid.

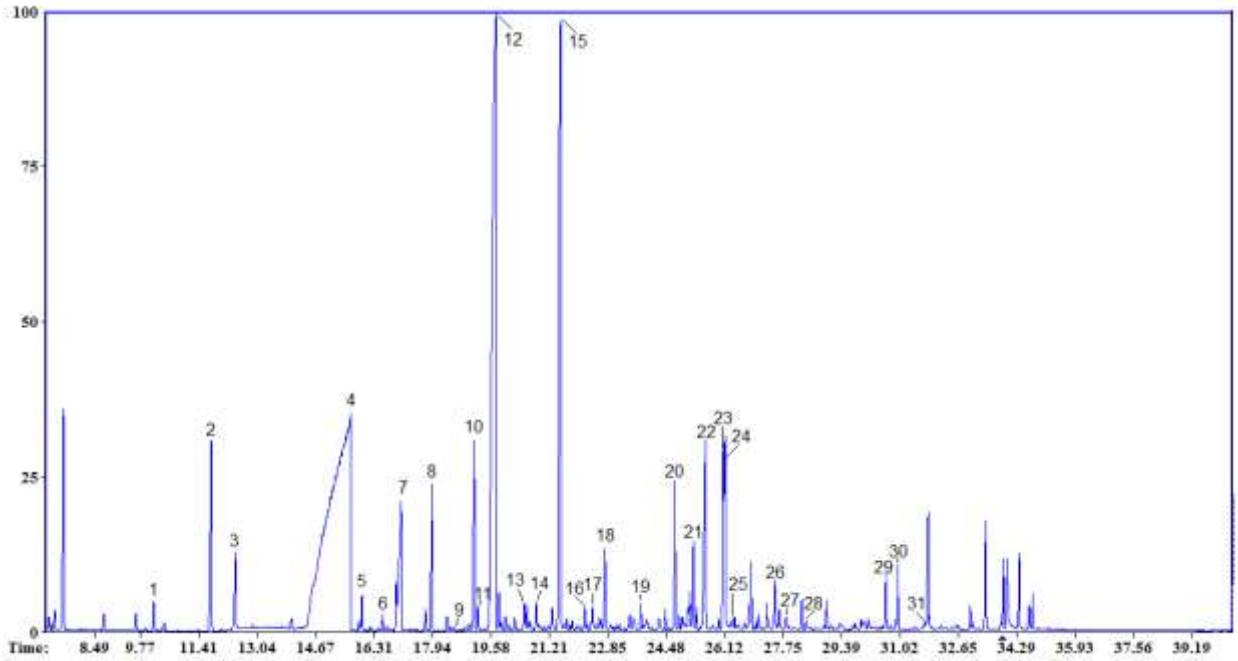


Table 1. Mean concentrations in mmol/L and standard deviations of the 30 most abundant organic acids detected in cheetah urine.

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

Figure 2. Mass spectrum of an unidentified compound (Unknown 362) detected at high concentration in the urine of captive cheetahs.

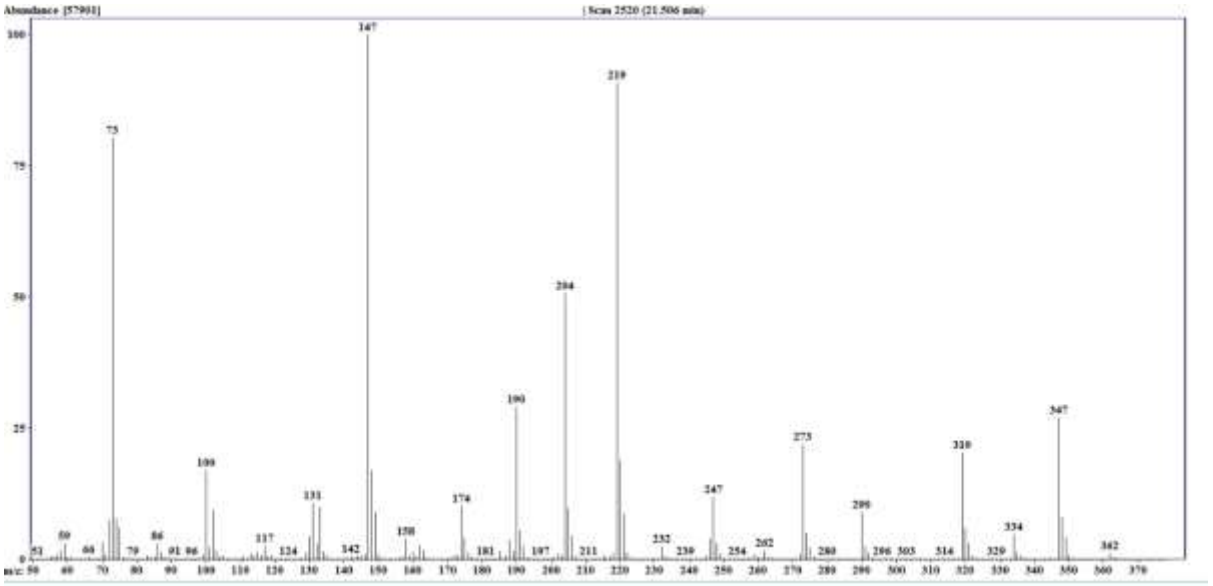
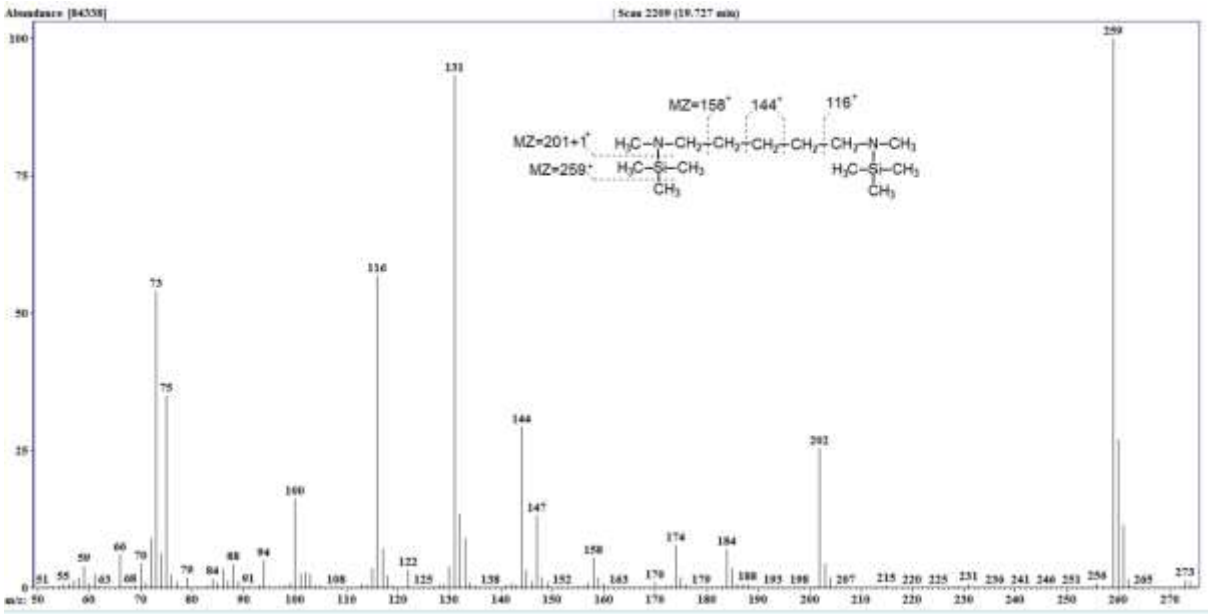


Figure 3. Mass spectrum and fragmentation of a compound detected at high concentration in the urine of captive cheetahs, tentatively identified as N1,N5-dimethylpentane-1,5-diamine.



Excretion of several metabolites including pantothenic acid, citramalic acid, 2 deoxy-3,5-dihydroxypentonic acid-γ-lactone, N-acetylisoleucine, ribose, 3-hydroxyphenylacetic acid and vanillylmandelic acid decreased significantly relative to age, while glutaric acid, 2-

hydroxyundecanoic acid and 1,2-dihydroxybenzene excretion were positively correlated with age (Table 2). Comparison of urine organic acid profiles between male and female cheetahs resulted in separation of the groups by the PLS-DA (Figure 4), but no separation in the PCA (Figure 5). A list of six metabolites responsible for the separation, with PLS-DA VIP scores > 3.0, Mann-Whitney $p \leq 0.05$ and effect size (ω) so that $|\omega| \geq 0.3$ was generated (Table 3).

Table 2. Urine organic acid concentrations that correlated with age (in years) in cheetahs (n = 41).

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

Figure 4. Two dimensional PLS-DA score plots of urinary organic acid profiles in male and female cheetahs.

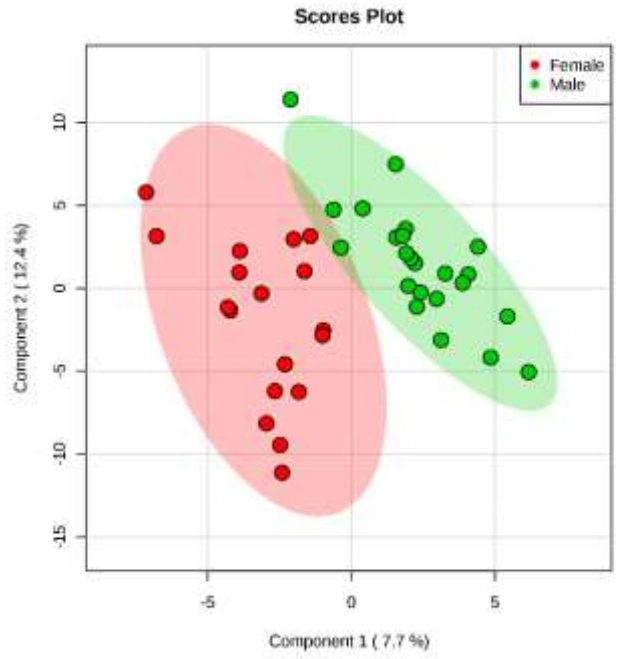


Figure 5. Two dimensional PCA score plots of urinary organic acid profiles in male and female cheetahs.

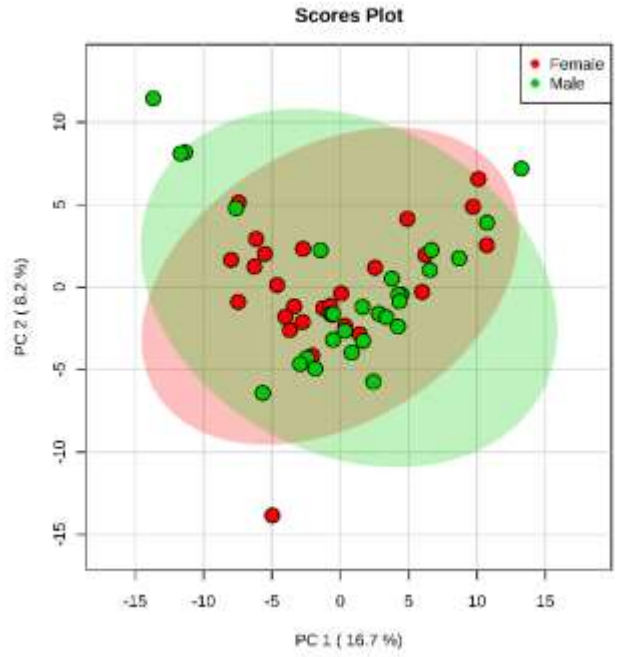
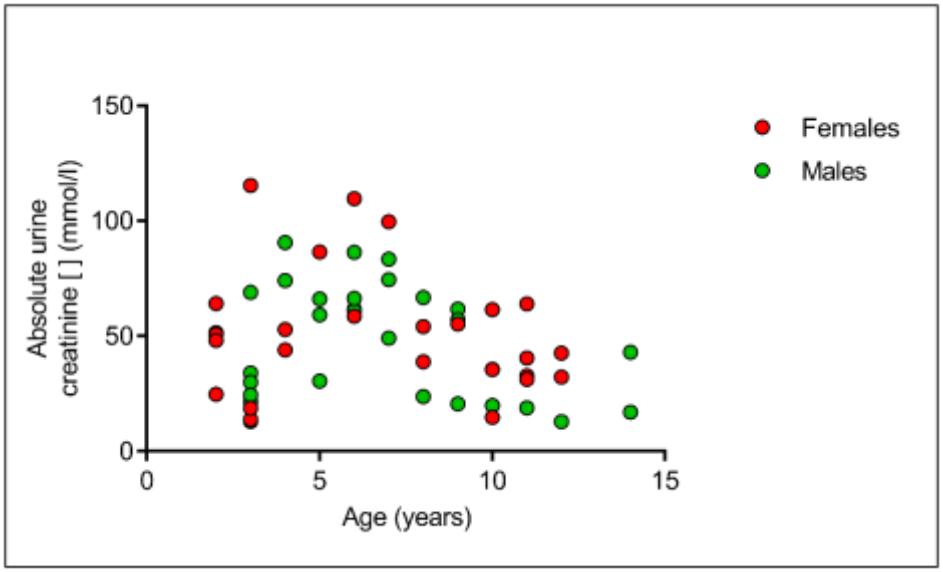


Table 3. Partial least squares discriminant analysis comparing the top VIP scores of urine organic acids in female and male cheetahs. PLS-DA VIP scores $> \mathbf{3.0}$, Mann-Whitney $\boldsymbol{p \leq 0.05}$ and effect size ($\boldsymbol{\omega}$) so that $\boldsymbol{|\omega| \geq 0.3}$

Metabolites	Female mean (mmol/L)	Male mean (mmol/L)	PLS VIP scores	Effect Size (ω-value)	p	Metabolic origin
N-Acetylphenylalanine	0.624	0.234	5.061	0.376	0.004	Enteric bacteria/Phenylalanine
2-Hydroxy-5-Methoxybenzoic acid	0.095	0.022	4.456	0.366	0.005	Unknown
N-Hexanoylglycine	10.32	4.63	4.140	0.338	0.010	Fatty acid metabolism
N-Tiglylglycine	0.880	0.304	4.123	0.303	0.021	Isoleucine metabolism
2,3-Dihydroxybenzoic acid	0.017	0.069	3.674	0.425	0.001	Enteric bacteria
Furoylglycine	0.057	0.017	3.459	0.316	0.016	Carbohydrate metabolism

The urinary creatinine concentrations did not differ between males and females, ranging from 12.9 mmol/L to 115.4 mmol/L with a mean of 48.64 mmol/L. Urine SG values ranged from 1.030 to 1.080 with a mean of 1.054 and were significantly higher in females than in males (p = 0.0173), but the mean difference was relatively small (0.007). Mean urine creatinine values initially increased relative to age, reaching a peak between three and seven years of age, after which they declined rapidly (Figure 6).

Figure 6. Scatterplot of absolute urine creatinine concentrations relative to age in male and female cheetahs.



Discussion

Almost a third of the thirty most abundant organic acids detected in the cheetah urine samples were phenolic compounds associated with the intestinal microbial fermentation of phenylalanine (4-hydrocinnamic acid) and tyrosine (4-hydroxyphenylacetic acid, 4-hydroxymandelic acid, and 4-hydroxyphenyllactic acid) or their corresponding glycine conjugates (N-phenylacetylglycine, hippuric acid, N-phenylpropionylglycine, 4-hydroxyphenylacetylglycine and cinnamoylglycine). Microbial fermentation of the aromatic amino acids has been shown in humans and laboratory rodents to occur primarily in the distal colon and results in the formation of a range of aromatic phenolic compounds, often referred to as xenobiotics, which are absorbed by the host and then modified by endogenous metabolism or excreted unchanged in the urine [35,36].

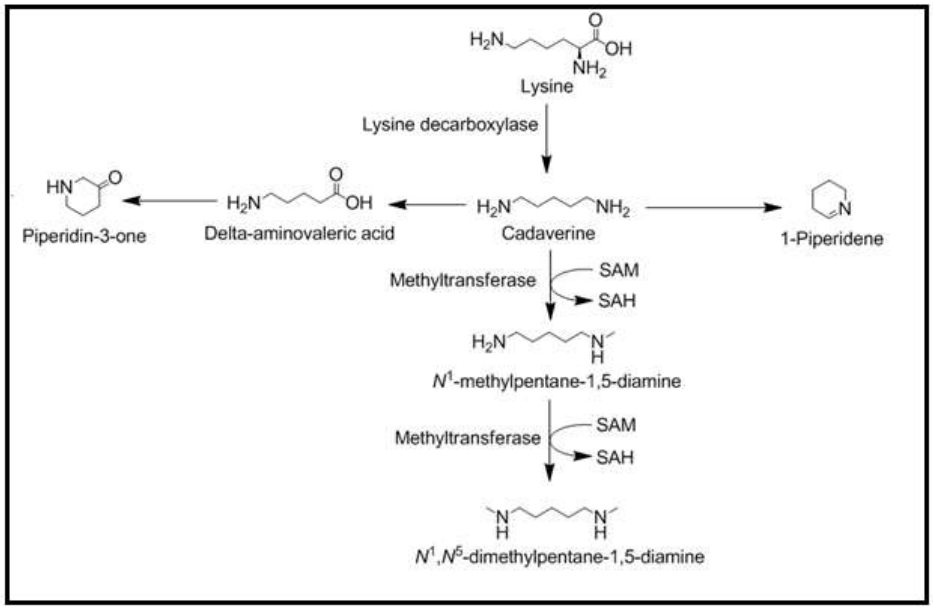
The absorption, conjugation and urinary excretion of microbial phenolic metabolites from amino acid precursors have been poorly studied in carnivores, and very little is known about their impact on metabolism and health. The provision of high-protein diets, rich in muscle meat, may provide significant quantities of undigested amino acids for colonic bacterial fermentation. Depauw *et al* compared the faecal excretion of short-chain- (SCFA) and branched-chain fatty acids (BCFA) as well as indole and phenol in cheetahs fed either supplemented beef or whole rabbits and found that on the whole-rabbit diet, cheetahs produced less BCFA, putrefactive indole and phenol [61]. They concluded that this decrease in putrefactive metabolites could be caused by the higher intake of poorly digestible tissue like skin, bone and collagen.

Amino acid conjugation in the mammalian hepatic and renal cells appears to be the primary method of aromatic phenolic compound metabolism prior to urinary excretion. This reaction takes place within the mitochondria in a two-step process which firstly involves the formation a xenobiotic acyl-coenzyme A (acyl-CoA) thioester followed by conjugation to glycine [37]. There is mounting evidence that many of the microbial phenolic compounds absorbed from the colon as well as the reactive acyl-CoA thioesters formed prior to conjugation are potentially toxic to the host organism (reviewed in [38]). For example, it has been demonstrated that, even at fairly low concentrations, the phenolic acids formed by bacteria during sepsis stimulate the production of reactive oxygen species (ROS) in isolated liver mitochondria and inhibit the activity of Complex 1 in the electron transport chain [39]. The acyl-CoA thioesters are reactive and able to modify proteins, inhibit enzymes and act as alternative substrates leading to the production of abnormal metabolites [37].

The two novel compounds, N^1,N^5-dimethylpentane-1,5-diamine and Unknown 362, detected at the highest concentrations in captive cheetah urine, have to our knowledge not been detected in the urine of any species before. The electron ionization pattern of Unknown 362 contains ion fragments of m/z 190, 204 and 219 (Figure 2), which are often seen in TMS-ethers of polyhydroxy compounds like sugar alcohols. The N^1,N^5-dimethylpentane-1,5-diamine is likely to be metabolised from cadaverine through transmethylation, as shown in Figure 7, in a similar way to the production of substances such as N,N-dimethyltryptamine (DMT) [40]. Cadaverine is a malodourous diamine produced by the bacterial decarboxylation of lysine [41] and has been shown to be mildly toxic to rats [42]. In captive cheetahs, the high protein intake from muscle meat, could potentially result in increased lysine being available for colonic bacterial decarboxylation. The potential toxic effects of cadaverine or its methylated derivative are unknown in the cheetah. The high concentrations, detected in the

urine of captive individuals, warrants further investigation of the potential toxicological effects of these compounds.

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



Since the incidence and severity of glomerulosclerosis, adrenal hyperplasia and gastritis correlate positively with age in captive cheetahs [8,43], we evaluated the association between urinary organic acid metabolites and the age of the cheetahs (Table 3). The three metabolites that correlated most strongly with age, namely pantothenic acid, citramalic acid and glutaric acid, are all in some way associated with CoA metabolism. Pantothenic acid, an important component of CoA and acyl carrier proteins, correlated negatively with age ($r = -0.408, p = 0.001$). This B vitamin is normally abundant in many food sources, mostly in the form of CoA, but concentrations in muscle meat are approximately 7 to 10 times lower than in organ meats such as liver and kidney [44]. Since dietary intake in captive cheetahs is, however, unlikely to change with age, reduced intestinal absorption or the increased

utilization of pantothenic acid could explain the reduced renal excretion of this vitamin in older cheetahs. An increased demand for CoA could reduce the available pool of pantothenic acid and therefore result in reduced renal excretion. Citramalic acid is a degradation product of itaconic acid in a process that also requires CoA [45] and lower concentrations in older cheetahs could therefore also be explained by a relative age-related CoA deficiency. Glutaric acid is an intermediate in the degradation pathway of tryptophan, lysine and hydroxylysine. In mammals the utilization of glutaric acid requires its mitochondrial activation to glutaryl-CoA, a reaction that once again necessitates CoA [46]. The role of potential CoA depletion in older cheetahs therefore requires further investigation.

The quantification of urinary metabolites is ideally done on the combined urine collected from an animal over a 24-hour period. Such 24-hour samples are impractical for obvious reasons in non-laboratory animals. Over the last few decades, creatinine excretion has most commonly been used to correct urine metabolite concentrations, as this breakdown product of creatine phosphate is thought to be produced at a fairly constant rate, is independent of diuresis and is excreted unchanged by the kidneys [47]. Concerns have, however, been raised about the universal use of creatinine excretion to correct urine metabolite concentrations [48]. Creatinine excretion in this population of cheetahs was highly variable with more than an eight-fold difference between minimum and maximum concentrations. Urine creatinine concentrations appeared to be largely influence by age, with a peak in excretion reached at between four and six years of age, followed by a decline with advancing age. Relying on urine creatinine to correct or "standardize" urine metabolite concentrations would therefore potentially result in the overestimation of metabolite concentrations in younger and older cheetahs. Variability in urine SG was considerably lower, as would be expected in a carnivorous mammal where water balance is tightly regulated [49]. In cheetahs, where dietary

protein intake is fairly similar between individuals, and where they are starved for the same period of time prior to sample collection, urine SG is in our view likely to provide a better indication of urine dilution and should therefore provide a more accurate means of correcting urine metabolite concentrations. Future studies should ideally evaluate the accuracy of various urine metabolite correction methods in this species.

Conclusion

It appears that a large proportion of the most abundant organic acids detected in the urine of captive cheetahs are the product of bacterial enteric amino acid fermentation. It is currently not clear if these metabolites are a normal feature of cheetah urine or the product excessive protein in the diet of captive individuals. Evidence from other studies, however, suggests that particularly the phenolic compounds and their acyl-CoA thioesters, formed prior to glycine conjugation could negatively impact the health of these animals [38]. Efforts have already been made to collect urine from a sufficient number of healthy free-ranging cheetahs for comparison. Dietary trials, combined with metabolomic and intestinal microbiome studies would also provide further important insights into these questions. This study importantly provides a baseline of data on the urine organic acids excreted by captive cheetahs and sets the stage for future investigations into the metabolism and health of these animals.

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