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REFERENCE INTERVALS FOR SELECTED SERUM BIOCHEMISTRY ANALYTES IN CHEETAHS (*ACINONYX JUBATUS*)

By

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Submitted in partial fulfilment of the requirements for the degree

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Dissertation

Reference intervals for selected serum biochemistry analytes in Cheetahs (*Acinonyx jubatus*)

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Declaration

I, Gavin Charles Hudson-Lamb, hereby declare that the research presented in this dissertation was conceived and executed by myself under guidance from my supervisors.

Neither the substance, nor any part of the dissertation has been submitted by me for a degree at this or any other tertiary institution, other than for the present degree for which I am enrolled.

This dissertation is presented in partial fulfillment of the requirements for the degree, Master of Science (Veterinary Science) under the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria.

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Gavin Hudson-Lamb

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List of Contents

ACKNOWLEDGEMENTS III

DECLARATION IV

LIST OF CONTENTS V

LIST OF TABLES VII

LIST OF FIGURES IX

LIST OF ABBREVIATIONS X

SUMMARY XIII

CHAPTER 1 1

LITERATURE REVIEW 1

1.1. Introduction 1

1.2. Prevalent Diseases in Captive Cheetahs 2

1.3. Reference Intervals in Cheetahs 4

1.4. The Effect of Haemolysis on Serum Analytes 5

CHAPTER 2 8

OBJECTIVES 8

2.1. Problem Statement 8

2.2. Benefits Arising from the Study 9

CHAPTER 3 10

MATERIALS AND METHODS 10

3.1. Experimental Design 10

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3.2. Experimental Procedures 12

3.3. Observations / Analytical Procedures 18

3.4. Data Analysis 18

CHAPTER 4 21

RESULTS 21

4.1. Study Population 21

4.2. Reference Intervals for Serum Analytes 23

4.3. Urine Analytes 31

4.4. Correlations between Variables 36

CHAPTER 5 37

DISCUSSION 37

5.1. Limitations of Study 55

5.2. Conclusion 57

REFERENCES 58

ADDENDUM A 65

RAW DATA 65

ADDENDUM B 68

SPEARMAN'S CORRELATION CO-EFFICIENTS 68

ADDENDUM C 69

JOURNAL PUBLICATION EMANATING FROM THIS RESEARCH 69

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List of Tables

Table 1: Co-efficient of variation (CV) of the analytes measured on the Cobas Integra 400 Plus Analyser®……………………………………….16

Table 2: Summary of sex and age distributions of captive and free-ranging cheetahs………………………………………………………………….21

Table 3: Reference intervals (RI) for seven serum analytes and serum ISIS values of cheetahs..…………………………………………………..25

Table 4: Statistics and RI for serum analytes for captive and free-ranging cheetahs, with *P*-values using the Mann-Whitney U test………….27

Table 5: Spearman's correlation co-efficients for measured serum analytes associated with age……………………………………………………..28

Table 6: MWU results for serum analytes between male and female cheetahs……………………………………………………………………29

Table 7: Haemolysis scores of serum samples between captive and free-ranging cheetahs………………………………………………………30

Table 8: Mean ± SD of serum analytes of all cheetahs, based on haemolysis score....……………………………………………………..31

Table 9: SG-corrected urine analytes in captive and free-ranging cheetahs…………………………………………………………………...32

Table 10: Comparison of SG-corrected urine sodium levels between captive and free-ranging cheetahs……………………………………...……32

Table 11: Spearman's correlation co-efficients for SG-corrected urine analytes associated with age…………………………………………….33

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YUNIBESITHI YA PRETORIA

Table 12: Spearman's correlation co-efficients between various serum and SG-corrected urine analytes of all cheetahs (correlation coefficients of $r_s > 0.6$ or < -0.6, $P < 0.05$)……………………………...…36

Table 13: Reference intervals for serum analytes in cheetahs in this study and domestic cats……………………………………………………………44

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List of Figures

Figure 1: Histogram of ages for captive and free-ranging cheetahs………..22

Figure 2: Histogram of serum urea concentration between captive and free-ranging cheetahs...…………………………………………………....28

Figure 3: Scatter plot of SG-corrected urine aldosterone based on age…..34

Figure 4: Scatter plot of SG-corrected urine sodium based on age..……….34

Figure 5: Scatter plot of SG-corrected urine creatinine based on age (r_s = 0.24)……………………………………………………………………..35

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List of Abbreviations

>	Greater than
<	Less than
°C	Degrees Celsius
ACTH	Adrenocorticotrophic Hormone
AEC	Animal Ethics Committee
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CI	Confidence Interval
CITES	Convention on International Trade in Endangered Species
CKD	Chronic Kidney Disease
Cl	Chloride
CK	Creatine Kinase
CLIA	Clinical Laboratory Improvement Amendment
CLSI	Clinical and Laboratory Standards Institute
Cr	Creatinine
CV	Co-efficient of Variation
DAFF	Department of Agriculture, Fishing and Forestry
Dr.	Doctor
e.g.	For example
et al.	And others
FE	Fractional Excretion
g	G-force
G	Gauge
GFR	Glomerular Filtration Rate
GGT	Gamma Glutamyltransferase

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Ha	Hectare
H_2O	Water
IFCC	International Federation of Clinical Chemistry
ISE	Ion-selective electrode
ISIS	International Species Information System
IUCN	International Union for Conservation of Nature
IZW	Leibniz Institute for Zoo and Wildlife Research
K	Potassium
kg	Kilogram
l	Litre
LDH	Lactate Dehydrogenase
Max	Maximum
mg	Milligram
Mg	Magnesium
Min	Minimum
ml	Millilitre
mmol	Millimole
µmol	Micromole
Mr.	Mister
Ms.	Miss
MWU	Mann-Whitney U Test
n	Number of samples
Na	Sodium
NZG	National Zoological Gardens of South Africa
OVAH	Onderstepoort Veterinary Academic Hospital
P	Phosphate
PhD	Philosophiae Doctor
pmol	Picomole
Prof.	Professor

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RAAS	Renin-Angiotensin-Aldosterone System
RESC	Research Ethics and Scientific Committee
Res Com	Research Committee of the University of Pretoria
RI	Reference Interval
RIA	Radio-immuno assay
SD	Standard Deviation
SG	Specific Gravity
UC	Urine Creatinine
UIBC	Unsaturated Iron Binding Capacity
UP	Urine Protein
UPC	Urine Protein: Urine Creatinine Ratio
VOD	Veno-occlusive Disease
WB	Wet Basis

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Summary

The cheetah (*Acinonyx jubatus*) is listed as vulnerable on the IUCN Red List of Threatened Species and is the last remaining member of the genus Acinonyx. Cheetahs in captivity have a high prevalence of three unusual diseases which are rare in the free-ranging population, as well as other large captive felids. These are chronic lymphoplasmacytic gastritis (often being associated with secondary amyloidosis), glomerulosclerosis and veno-occlusive disease.

The measurement of blood and urine analytes plays a major role in the assessment of the health of an individual or population. Species-specific reference intervals are an important tool in the clinical decision-making process and aid in the diagnosis and management of disease. There is very little published information on reference intervals for serum biochemistry analytes in captive and free-ranging cheetahs. However, information on haematological and serum biochemistry analytes are available for captive cheetahs on the International Species Information System (ISIS) database but these are not published reference intervals for healthy cheetahs. Published data on urine electrolyte concentrations and biochemistry analytes, as well as the effect of haemolysis on cheetah serum samples does not currently exist.

The aim of this study, therefore, was to establish reference intervals for healthy cheetahs for commonly measured serum electrolyte concentrations and renal physiological analytes according to international guidelines from the International Federation of Clinical Chemistry (IFCC) and the Clinical and Laboratory Standards Institute (CLSI). The effect of age, sex, haemolysis and captivity status on these

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analytes was also investigated. Urine electrolyte concentrations and biochemistry analytes were also measured and described.

The study population consisted of 75 cheetahs, of which 36 were captive cheetahs and 39 were free-ranging cheetahs. During June – July 2013, the captive cheetahs were immobilised and sampled at the AfriCat Foundation, Okonjima, Namibia, during their annual veterinary health examinations. The free-ranging cheetahs were trapped, immobilised and sampled on communal and commercial farmland in the Khomas and Omaheke districts, central Namibia. Animals that were used in the study were clinically healthy as far as could be determined according to history and clinical examination.

Baseline serum biochemistry analytes were analysed from 30 captive cheetahs and 36 free-ranging cheetahs. Baseline urine biochemistry analytes were analysed from 37 cheetahs, of which 31 were captive cheetahs and six were free-ranging cheetahs. Serum and urine samples were obtained within 15 minutes of immobilization.

Serum analytes that were measured included sodium, potassium, magnesium, chloride, urea, creatinine and osmolarity. The 90% confidence interval of the reference limits was obtained using the non-parametric bootstrap method. Reference intervals were preferentially determined by the non-parametric method. Urine analytes that were measured were sodium, potassium, magnesium, chloride, uric acid, creatinine, urine protein, urine protein: urine creatinine ratio, aldosterone, osmolarity and urine SG. All urine analytes, excluding osmolarity, were adjusted and corrected relative to urine SG. The age, sex and body weight of the cheetahs was also documented. Haemolysis scores of the serum samples were described by visual inspection.

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The reference intervals calculated are: sodium (128 – 166 mmol/l), potassium (3.9 – 5.2 mmol/l), magnesium (0.8 – 1.2 mmol/l), chloride (97 – 130 mmol/l), urea (8.2 – 25.1 mmol/l), creatinine (88 – 288 µmol/l) and osmolarity (293 – 356 mOsmol/l). The mean ISIS values for those analytes are within the calculated reference intervals. Separate reference intervals for captive and free-ranging cheetahs were also determined. The median urea concentration of captive cheetahs (15.7 ± 3.7 mmol/l) was significantly higher than that of free-ranging cheetahs (11.4 ± 3.5 mmol/l) (MWU test, $P < 0.01$). This is suspected to be due to the high protein diet which the captive cheetahs were fed. No separate reference intervals were calculated for the sub-groups based on age and sex. There were no significant changes observed in serum analytes as a result of haemolysis. Descriptive statistics for the urine analytes are described. However, no reference intervals and statistical comparisons between captive and free-ranging cheetahs could be performed due to small sample size.

The reference intervals for the serum analytes measured in this study can be used by veterinary practitioners, researchers, zoologists and any other profession to aid in the clinical decision-making process of establishing diagnoses and the management of disease, particularly renal disease in cheetahs. The data obtained for the urine analytes can also be used to aid in the assessment of the health status of captive and free-ranging cheetahs.

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CHAPTER 1

Literature Review

1.1. Introduction

The cheetah (*Acinonyx jubatus*) is the last remaining member of the genus Acinonyx (Bolton & Munson 1999) and is listed as vulnerable on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN 2014). In an attempt to ensure the survival of the species, many cheetahs are being kept in captivity to encourage breeding (Carstens et al. 2006). However, captive cheetahs have poor reproductive performance and reduced longevity compared to other large felids in captivity (Munson 1993). The future of cheetahs in captivity, therefore, depends on reversing these trends of low fecundity and shortened lifespans. In order to achieve this, the causes for higher mortality and reduced fertility in captive cheetah populations should be better understood before strategies can be developed to improve captive breeding and management of cheetahs.

It has been postulated that genetic homogeneity, management conditions, behaviour and dietary factors are underlying causes of low rates of reproductive success in North American captive cheetah populations (Bechert et al. 2002; Munson et al. 2005).

The reduced longevity of cheetahs in captivity has been attributed to the high prevalence of three unusual diseases: chronic lymphoplasmacytic gastritis (often

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being associated with secondary amyloidosis), glomerulosclerosis and veno-occlusive disease (VOD) (Munson 1993; Munson et al. 1999). These diseases are uncommon in other captive wild felids (Munson et al. 1999; Newkirk et al. 2011) and have only rarely been documented in free-ranging Namibian and South African cheetahs (Terio, Marker & Munson 2004). The cause for the high disease prevalence in captive cheetahs has not been elucidated, but is suspected to be due to a combination of chronic stress, lack of exercise, genetic homogeneity and dietary factors (Bolton & Munson 1999; Munson 1993; Terio, Marker & Munson 2004).

1.2. Prevalent Diseases in Captive Cheetahs

Gastritis has a prevalence of 11% in free-ranging Namibian cheetahs, compared to 99% in both captive South African and North American cheetahs (Munson et al. 2005). Gastritis and its sequelae [asphyxiation or pneumonia due to aspirated food material, and systemic (mainly renal) amyloidosis] account for 29% of mortality in North American captive cheetahs and 40% in South African captive cheetahs, warranting intensive research on its pathogenesis and control (Munson 1993; Munson et al. 1999). Gastritis occurs in captive cheetahs worldwide in a variety of management systems and it has been found that at least 95% of cheetahs with gastritis have concurrent *Helicobacter* and *Helicobacter*-like organisms, indicating that these gastric spiral bacteria play a role in the pathogenesis of gastritis (Munson 1993; Munson et al. 1999; Terio et al. 2004). However, about 25% of South African captive cheetahs had gastritis without spiral bacteria, a finding that has only rarely been documented in North American captive cheetahs (Munson et al. 1999).

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Furthermore, some captive cheetahs and most free-ranging cheetahs have spiral bacteria but do not show significant gastritis (Munson et al. 2005; Terio et al. 2004). This indicates that the pathogenesis of gastritis is multifactorial. An auto-immune component to gastritis is possible, because the inflammatory reaction is predominantly lymphoplasmacytic and orientated towards gastric glands (Munson et al. 1999). Other possible causes of lymphoplasmacytic gastritis, such as duodenal reflux, idiopathic hypertrophy and infectious agents, are rarely diagnosed in captive cheetahs (Lane et al. 2012).

Renal disease is a leading cause of morbidity and mortality in captive cheetahs (Munson 1993), and renal lesions were present in 90% of a captive cheetah population in one study (Munson 1993). Glomerulosclerosis and renal amyloidosis are the two main pathological lesions seen in captive cheetahs with renal disease (Munson 1993; Papendick et al. 1997). The lesion causing glomerulosclerosis resembles the glomerulopathy of early onset diabetic nephropathy of humans or chronic progressive nephropathy of rats (Bolton & Munson 1999). It has been postulated that stress compounded by a genetic predisposition and daily feeding of high protein diets may play a role in the pathogenesis, however, the exact cause of glomerulosclerosis has not yet been elucidated (Bolton & Munson 1999). With regards to renal amyloidosis, amyloid fibrils are deposited in the renal medullary interstitium, resulting in localised ischaemia and ultimately coagulation necrosis of the renal papilla and pyelonephritis (Papendick et al. 1997). Ischaemic atrophy and necrosis of tubules results in loss of nephrons, with secondary glomerulosclerosis and cortical atrophy, the end result being chronic renal failure (Papendick et al. 1997). Other causes of renal failure in captive cheetahs include oxalate nephrosis, nephrosclerosis, glomerulonephritis, chronic interstitial nephritis, membranous

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glomerulopathy, renal papillary nephritis and pyelonephritis (Bolton & Munson 1999; Munson 1993).

Veno-occlusive disease is the disease process whereby fibrosis surrounds or occludes hepatic sinusoids and central and/or sub-lobular veins of the liver (Munson 1993). VOD is widespread in the North American captive cheetah population (63%) and is less prevalent in the South African captive cheetah population (43%) (Munson 1993; Munson et al. 2005). It is also less severe, 16% being moderate to severe cases of VOD in South African captive cheetahs, compared to 43% in North American cheetahs (Munson et al. 1999). The notable increase in severity of VOD with age in both live and deceased cheetahs suggests that VOD is a progressive disease (Munson 1993). The severe cases of VOD in South African captive cheetahs occurred in city zoos and not in rural breeding facilities, implicating stress and environmental factors in the pathogenesis of this disease (Munson et al. 1999).

1.3. Reference Intervals in Cheetahs

Reference intervals have gained universal acceptance in human medicine and have subsequently been applied to veterinary species. They are arguably one of the most powerful tools in laboratory medicine, where they aid in making diagnoses and in the management of disease (Friedrichs et al. 2012). Reference intervals describe acceptable or normal fluctuations observed in healthy populations or individuals. The definition of a healthy population or individual is thus a critical step in assessing the health of animals or a population (Friedrichs et al. 2012).

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Published haematologic and serum biochemistry reference intervals are very scarce for captive cheetahs, and more so for free-ranging cheetahs. In addition, these reference values are only listed as the mean ± standard deviation (Bechert et al. 2002; Caro et al. 1987; Depauw et al. 2012; Holder et al. 2004) and, therefore, do not comply with the guidelines stipulated by the International Federation of Clinical Chemistry (IFCC) and the Clinical and Laboratory Standards Institute (CLSI) regarding the generation of reference intervals for veterinary species (Friedrichs et al. 2012). Unpublished data on haematologic and serum biochemistry analytes, in the form of basic descriptive statistics, are also available on the International Species Information System (ISIS) (ISIS 2002). These values form part of an electronic database of cheetahs held only in zoological institutions, to which member institutions provide health and genetic data (ISIS 2002). No information about health status is available for the ISIS values, and they may include multiple samples per animal (ISIS 2002). Furthermore, there are no published data on the urine electrolyte and biochemistry analytes of cheetahs.

1.4. The Effect of Haemolysis on Serum Analytes

Haemolysis may occur either in vivo or in vitro and is a common cause for unreliable and inaccurate results obtained from serum samples for chemical analysis (Lippi et al. 2011). Haemolysis can cause three different changes to sample results. Firstly, the release of haemoglobin and other intracellular components from erythrocytes can result in increased values of certain serum analytes (Frank et al. 1978, Lippi et al. 2011). Secondly, the effect of dilution can result in decreased values (Frank et al.

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1978, Lippi et al. 2006). Thirdly, haemoglobin may interfere directly with the analytical quantification of some analytes (Frank et al. 1978, Lippi et al. 2006). The interference caused by haemolysis seems to be related to the degree of lysis of the erythrocytes and the specificity of the analytical method being used, with several laboratory results being severely affected even by slight degrees of sample haemolysis (Lippi et al. 2006).

Lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) are affected at almost undetectable haemolysis by visual inspection (plasma haemoglobin < 0.5 g/l) (Koseoglu et al. 2011). Clinically meaningful variations of potassium and total bilirubin are observed in moderately haemolysed samples (haemoglobin > 1g/l) (Koseoglu et al. 2011). Alanine aminotransferase (ALT), cholesterol, gamma glutamyltransferase (GGT) and inorganic phosphate (P) concentrations are not interfered with up to severely haemolysed levels (haemoglobin: 2.5-4.5 g/l) (Koseoglu et al. 2011, Yücel & Dalva 1992). The degree of bias as a result of haemolysis for albumin, alkaline phosphatase (ALP), amylase, calcium, chloride, HDL-cholesterol, creatine kinase (CK), creatinine, glucose, iron, magnesium, sodium, total protein, triglycerides, unsaturated iron binding capacity (UIBC), urea and uric acid are lower than the Clinical Laboratory Improvement Amendment's (CLIA) allowable limits even in severely haemolysed samples (Koseoglu et al. 2011, Yücel & Dalva 1992).

Three approaches can be applied to deal with haemolysed samples: haemolysis correction, result reporting with a clear indication of the potential interference arising from erythrocyte lysis and sample recollection (Lippi et al. 2006).

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In summary, the effect of haemolysis on serum analytes of cheetahs has not been investigated. The aim of this study, therefore, was firstly to establish reference intervals for healthy cheetahs for commonly measured serum electrolyte concentrations and renal physiological analytes according to international guidelines from the IFCC and the CLSI. Secondly, the effect of age, sex, haemolysis and captivity-status on these analytes was also investigated. Finally, urine electrolyte concentrations and biochemistry analytes were measured and described.

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CHAPTER 2

Objectives

2.1. Problem Statement

The IUCN Red List of Threatened Species classifies cheetahs as vulnerable (IUCN 2014). Captive cheetahs have reduced longevity and this has been attributed to the high prevalence of three diseases, namely: chronic lymphoplasmacytic gastritis (often being associated with secondary amyloidosis), glomerulosclerosis and veno-occlusive disease (Munson 1993, Munson et al. 1999). Species-specific reference intervals aid in the diagnosis and management of disease (Friedrichs et al. 2012). There is very little published information on reference intervals for serum biochemistry analytes in captive and free-ranging cheetahs. ISIS provides data on haematological and serum biochemistry analytes for captive cheetahs, but these are not published reference intervals for healthy cheetahs (ISIS 2002). In addition, published data on urine electrolyte concentrations and biochemistry analytes does not currently exist.

In order to detect disease in cheetahs, particularly captive cheetahs, the establishment of reference intervals for haematological and serum biochemistry analytes in healthy cheetahs, according to international guidelines stipulated by the International Federation of Clinical Chemistry (IFCC) and the Clinical and Laboratory Standards Institute (CLSI), needs to be performed.

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2.2 Benefits Arising from the Study

The information obtained from this study will enable the veterinary profession, any facility housing captive and/or free-ranging cheetahs, and any other interested parties to have access to the reference intervals for selected serum biochemistry analytes of cheetahs. It will also contribute to the accurate assessment of health status, the development of screening programmes and the diagnosis of disease in individual cheetahs or within a population. Results from this study will provide essential information for future investigations into the causes of renal disease and/or other diseases in cheetahs, therefore, leading to better conservation of the species.

This study is the basis of a postgraduate MSc degree in Veterinary Science, undertaken by Gavin Hudson-Lamb through the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria.

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CHAPTER 3

Materials and Methods

3.1. Experimental Design

This is a study aimed at establishing reference intervals for several serum biochemistry analytes in cheetahs; namely sodium, potassium, magnesium, chloride, urea, creatinine and osmolarity. Urine electrolyte concentrations (sodium, potassium, magnesium and chloride) and other urine biochemistry analytes (urine SG, uric acid, creatinine, urine protein, urine protein: urine creatinine ratio, aldosterone and osmolarity) were described for captive and free-ranging cheetahs. These serum and urine analytes were chosen for analysis in this study in order to focus on cheetah renal physiology since renal disease is a leading cause of morbidity and mortality in captive cheetahs (Munson 1993). In addition, the limited volume of the serum and urine samples that were obtained and project cost constraints restricted the number of analytes that could be analysed.

The study population consisted of 75 cheetahs (*Acinonyx jubatus*), of which 50 were males and 25 were females. The captive cheetahs (n = 36) belonged to the AfriCat Foundation in Namibia. The husbandry (housing/enclosures, diet, health management, veterinary care and treatment) of these cheetahs is the same. They are kept either singly or in small groups in large camps, 5 – 25 Ha in size, and are fed 1.0 – 1.5 kg of donkey meat per day. The free-ranging cheetahs (n = 39) in this

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study are cheetahs that roam freely on communal and commercial farmland in central Namibia. These cheetahs hunt for themselves and have access to natural prey consisting of small- to medium-sized antelope species.

Animals that were selected for the study were clinically healthy as far as could be determined according to history (previous disease and/or veterinary intervention and treatment administered) and clinical examination (body condition score; body mass index; rectal temperature; heart rate; respiratory rate; systolic, diastolic and mean arterial pressure measurements using a non-invasive high-definition oscillometric device at the coccygeal artery).

Approval for this study was obtained from the following committees:

- University of Pretoria
  - Research Committee (Res Com) (V033/13)
  - Animal Ethics Committee (AEC) (V033/13)

- National Zoological Gardens of South Africa's Research Ethics and Scientific Committee (RESC) (NZG/P13/26)

- Namibian Ministry of Environment and Tourism (1846/2013, 1689/2012 and 1813/2013)

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3.2. Experimental Procedures

Thirty frozen serum and 31 frozen urine supernatant samples from 36 captive cheetahs at the AfriCat Foundation; and 36 frozen serum and six frozen urine supernatant samples from 39 free-ranging cheetahs from central Namibia were used in this study to measure the various serum and urine biochemistry analytes. Access to these samples was made possible by Dr. Adrian Tordiffe of the National Zoological Gardens of South Africa (NZG).

The captive cheetahs were immobilised by remote intramuscular injection for their annual veterinary health examinations during June – July 2013 by Dr. A. Tordiffe. The free-ranging cheetahs were captured in box traps placed at cheetah marking trees and then immobilised by remote intramuscular injection by Drs. B. Wachter and S. Heinrich of the IZW. Immobilisation was achieved with a combination of 0.03 mg/kg medotomidine (Medotomidine - 10 mg/ml, Kyron Laboratories, Johannesburg, South Africa, 2094) and 1.2 mg/kg zolazapam/tiletimine (Zoletil®, Virbac Animal Health, Centurion, South Africa, 0157) for the captive animals, and 0.08 mg/kg medetomidine and 4.5 mg/kg ketamine (Ketamine 1G, Kyron Laboratories, Johannesburg, South Africa, 2094) for the free-ranging animals. Free-ranging cheetahs were reversed with 0.25 mg/kg atipamezole and released again.

Serum samples were obtained within 15 minutes of immobilisation. For the captive cheetahs, 10 ml of whole blood was collected from the jugular vein with a 20 ml syringe and 18 G needle. The blood was then transferred into serum BD Vacutainer® tubes (Becton, Dickinson and Company, Woodmead, Johannesburg, South Africa, 2191) and allowed to clot for 40 minutes on ice. After centrifuging the samples at

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1700 g for five minutes, the serum was pipetted off into 1.8 ml Cryovials® (Thermo Scientific, Germiston, South Africa, 1401) and frozen at -20 °C. In the free-ranging cheetahs, blood was collected directly into serum BD Vacutainer® tubes. The samples were placed on ice for between 4 and 24 hours until they could be centrifuged at 400 g for 15 minutes. The serum was then separated from the cells and frozen at -20 °C.

Haemolysis scoring of the serum samples was done by means of visual inspection and scored as 0 (no haemolysis), 1+ (mild haemolysis) and > 1+ (moderate – severe haemolysis) after centrifugation was performed. Ideally, the degree of haemolysis of serum samples should be quantified by measuring the haemoglobin content of the sample. In this study, the degree of haemolysis was assessed visually due to cost constraints. This is not the most accurate method to determine the amount of haemolysis as visual inspection is a subjective measure.

Samples remained frozen until time of transportation where they were transported in a liquid nitrogen canister to the NZG, Pretoria, South Africa. The immobilisation drugs and doses used to immobilise the cheetahs as well as the collection, handling and centrifugation of the serum samples differed between the captive and free-ranging cheetahs due to different researchers collecting and processing the samples. The lack of standardisation was as a result of this study being drafted and performed after the samples were collected.

Urine samples were collected within 15 minutes of immobilisation by catheterisation of the urethra with a 6 FG dog urinary catheter using aseptic technique into 15 ml sterile plastic syringes. Urine samples were aliquotted into 1.8 ml Cryoviles® (Thermo Scientific, Germiston, South Africa, 1401) and immediately frozen at -20 °C

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until the time of transportation. Samples were transported frozen in a liquid nitrogen canister to the NZG, Pretoria, South Africa. Due to the fact that there is a variable volume of urine present in the bladder of a particular cheetah at any particular point in time and that cheetahs commonly express their bladders upon immobilisation, it was not possible to collect the same required volume of urine from each cheetah. As a result, there was insufficient urine obtained from most of the cheetahs to measure all of the urine analytes in this study. Urine analytes were given the following preference (from highest to lowest) for analysis: urine SG, creatinine, electrolytes (sodium, potassium, magnesium and chloride), uric acid, urine protein, osmolarity and aldosterone.

The Convention on International Trade in Endangered Species (CITES) permit and the necessary veterinary export/import permits were obtained from the Department of Agriculture, Fishing and Forestry (DAFF) for the transport of the samples from Namibia to the NZG, Pretoria, South Africa. The samples were stored at -80 °C at the NZG until they were analysed. The samples were transported on dry ice to the various laboratories where the sample analysis was performed.

During sample collection, the cheetahs were weighed and the age and sex of the cheetahs was recorded. The age of the captive cheetahs was transcribed from accurate records that were available at the AfriCat Foundation. The age of the free-ranging cheetahs was estimated by the researchers of the IZW using the key for body size established by Caro (1994) for East African cheetahs. This was performed by evaluating shoulder height, appearance of the mane, dental wear and physical lesions such as elbow calluses and scars.

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The following analytes were analysed at the Clinical Pathology Laboratory at the Onderstepoort Veterinary Academic Hospital (OVAH), Faculty of Veterinary Science, University of Pretoria:

> Serum: sodium (Na), potassium (K), magnesium (Mg), chloride (Cl), urea and creatinine (Cr).
>
> Urine: sodium, potassium, magnesium, chloride, uric acid and urine protein: urine creatinine ratio (UPC).

The Cobas Integra 400 Plus Analyser® (Roche, Illovo, Johannesburg, South Africa, 1609) was used to measure the above-mentioned serum and urine analytes. Both daily internal and monthly external quality control was performed on this analyser and results fell within the laboratory's pre-set performance goals.

The quantitative determination of serum sodium, potassium and chloride was achieved using ion-selective electrodes (ISE indirect method). Serum magnesium concentrations were determined using a colorimetric method with Chlorophosphonazo III. Serum urea determinations were performed by a kinetic test with urease and glutamate dehydrogenase. Serum creatinine concentrations were measured using a buffered kinetic Jaffé reaction without deproteinisation. Haemolysis does not affect the analytical quantification of these serum analytes using the methods of analysis mentioned above (Hübl et al. 1994, Koseoglu et al. 2011), except for potassium where the potassium values are in direct proportion to the increase in plasma free haemoglobin concentration (Koseoglu et al. 2011).

Measures for analytical variation for the analytes tested are given below (Friedrichs et al. 2012) and fall within the performance goals of the laboratory.

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Table 1. Co-efficient of variation (CV) of the analytes measured on the Cobas Integra 400 Plus Analyser®

Analyte	Control concentration 1 (mmol/l)	Between-run CV (%)	Control concentration 2 (mmol/l)	Between-run CV (%)
Sodium	114	1.2	137	1.3
Potassium	3.7	1.1	6.5	1.2
Magnesium	0.81	2.5	1.3	2.3
Chloride	80.1	1.8	106	1.9
Urea	6.5	3.1	20.7	2.4
Creatinine	94	3.4	345	2.4

Urine sodium, potassium and chloride concentrations were determined using ion-selective electrodes (ISE indirect method) with automatically diluted urine specimens. Urine magnesium concentrations were determined by using a colorimetric method with Chlorophosphonazo III. An enzymatic colorimetric test was used for uric acid quantification and a turbidimetric method was used for urine protein measurements.

Urine creatinine was analysed at the North-West University by Dr. A. Tordiffe as part of his PhD study and was determined using an enzymatic method on the Indiko Clinical Chemistry Analyser® (Thermo Scientific, Germiston, South Africa, 1401).

Serum and urine osmolarity were measured with the Roebling Micro-Osmometer® (Automatic), Type 13/13DR-Autocal by the Physiology Laboratory, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria. The calibration standard used was 300 mOsmol/kg NaCl/H_2O and distilled water.

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The Ampath Laboratory in Centurion, Gauteng, South Africa analysed urine aldosterone using the Wizard Gamma Counter® (PerkinElmer South Africa (Pty) Ltd, Midrand, South Africa, 1644) by means of the radio-immuno assay (RIA) method.

Urine SG was measured and recorded with a hand-held refractometer (RHC-200) after urine samples were thawed and allowed to warm to room temperature. The refractometer was calibrated with distilled water after every 20 samples.

All of the urine analytes except for urine osmolarity were adjusted, corrected and presented relative to urine SG. The procedure for specific gravity normalisation of urine analyte concentrations was based on the Levine-Fahy equation (Cone et al. 2009) as follows:

$$[\text{Corrected}] = \frac{[\text{Measured}] \times (\text{Reference SG} - 1)}{(\text{Measured SG} - 1)}$$

where the urine analyte concentration was expressed in the relevant unit of measurement, Reference SG was a population reference value for specific gravity representing ‘normal’ urine SG and Measured SG was the specific gravity of the test specimen. The Reference SG used in this study was the mean urine SG of the study population, which was 1.053.

Urine protein: urine creatinine ratios were calculated using the following formula:

$$\text{UPC} = \text{UP}/\text{UC} \times 8.8$$

where UP was measured in g/l, UC was measured in mmol/l and 8.8 is the conversion factor for creatinine from mmol to grams. The molar mass of creatinine is

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113 g/mol, i.e. 0.113 g/mmol. There are 8.8 mmol in 1 g of creatinine (1 g ÷ 0.113 g = 8.8).

3.3. Observations / Analytical Procedures

The following data were collected, described and analysed for each study animal (where applicable):

i. Signalment: captive / free-ranging, age, sex and weight.

ii. Serum biochemistry: sodium, potassium, magnesium, chloride, urea, creatinine, serum osmolarity and haemolysis score.

iii. Urinalysis: SG, sodium, potassium, magnesium, chloride, uric acid, creatinine, aldosterone, UPC and urine osmolarity.

3.4. Data Analysis

All serum data were analysed using the Reference Value Advisor®, Version 2.1 (for Microsoft Office 2010) (Geffré et al. 2011). The selection of computations performed was guided by recommendations for the generation of reference intervals in veterinary species published by the American Society for Veterinary Clinical Pathology (ASVCP) (ASVCP 2015), based in turn on guidelines from the IFCC and the CLSI (Friedrichs et al. 2012). Common descriptive statistics for all serum

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analytes included sample size, mean, median, standard deviation (SD), and minimum (min) and maximum (max) values. The data were examined visually using histograms. The Dixon-Reed and Tukey's tests were used to test for and identify outliers, and emphasis was on retaining rather than deleting outliers. The variables were tested for Gaussian (normal) distribution according to the Anderson-Darling test with a significance level of 5%, as well as by visual inspection using histograms and Q-Q plots. The 90% Confidence Interval (CI) of the reference limits was obtained using the non-parametric bootstrap method. Reference intervals (RI) were preferentially determined by the non-parametric method, which is independent of the distribution of the data. Where non-parametric methods could not be used, reference intervals were based on a robust method, preferably after Box-Cox transformation of the data to a distribution that was Gaussian or symmetrical.

The Mann-Whitney U (MWU) test was used to determine any significant difference between the captive and free-ranging cheetah populations with regard to the age distribution and the serum analytes. Chi-Square tests were performed for sex and haemolysis scores to determine significant differences between the captive and free-ranging cheetahs. To determine if there was an association between the age, sex or haemolysis score with serum biochemistry test results, the Spearman's rank correlation co-efficients were calculated for age, the MWU test for sex, and the Kruskal-Wallis test for haemolysis score. *P*-values < 0.05 and Spearman's correlation co-efficient of $r_s > 0.6$ or < -0.6 were considered significant (Weir n.d.). Additional reference intervals were calculated for any such significant associations or differences.

No comparative statistics, specifically the MWU test, could be performed on the urine data between the captive and free-ranging cheetahs due to the small sample sizes.

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However, common descriptive statistics for all urine analytes were performed and included sample size, mean, median, standard deviation, and minimum and maximum values. To determine if there was an association between age or sex within urine biochemistry test results, Spearman's rank correlation co-efficients were calculated for age and the MWU test was applied for sex. *P*-values < 0.05 and Spearman's correlation co-efficient of $r_s > 0.6$ or < -0.6 were considered significant (Weir n.d.).

The statistical software that was used to analyse the serum and urine data were SAS®; Version 9.3 and Statistica®; Version 12, as well as the Reference Value Advisor (Geffré et al. 2011) add-on for Microsoft Excel. The data analysis was performed by the primary researcher with assistance from the project supervisor and co-supervisor, Dr. Adrian Tordiffe and Prof. Johan Schoeman respectively; as well as Dr. Emma Hooijberg. Staff from the Department of Statistics, University of Pretoria, Ms. Jaqui Sommerville and Mr. Paul van Staden, aided in the statistical analysis of the data.

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CHAPTER 4

Results

4.1. Study Population

The study population consisted of 75 captive and free-ranging cheetahs (*Acinonyx jubatus*). The captive cheetahs (n = 36) consisted of 20 male and 16 female cheetahs between the ages of four to fourteen years of age. The free-ranging cheetahs (n = 39) consisted of 30 male and nine female cheetahs between the ages of three months and eight years of age.

Statistically, the Chi-Square test demonstrates that there were significantly more male cheetahs in the free-ranging than the captive population, where $X^2 = 3.8$ (degrees of freedom = 1) and $P = 0.0499$.

A summary of the sex distribution of the study population is given in Table 2. From this table it can be seen that 76.9% (30/39) of the free-ranging cheetahs were male, whereas 55.6% (20/36) of the captive cheetahs were male. Two thirds (66.7%) of the total study population were male.

Table 2: Summary of sex distributions of captive and free-ranging cheetahs

	Captive (n = 36), %	Free-ranging (n = 39), %
Sex		
Male	20 (55.6)	30 (76.9)
Female	16 (44.4)	9 (23.1)

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Figure 1 depicts the age distribution of the captive and free-ranging cheetahs. The MWU test showed that the captive cheetahs were significantly older ($P < 0.001$), where the median age of the captive cheetahs was seven years (Inter-quartile range: 5 – 11 years), compared to four years (Inter-quartile range: 2 – 6 years) for the free-ranging cheetahs.

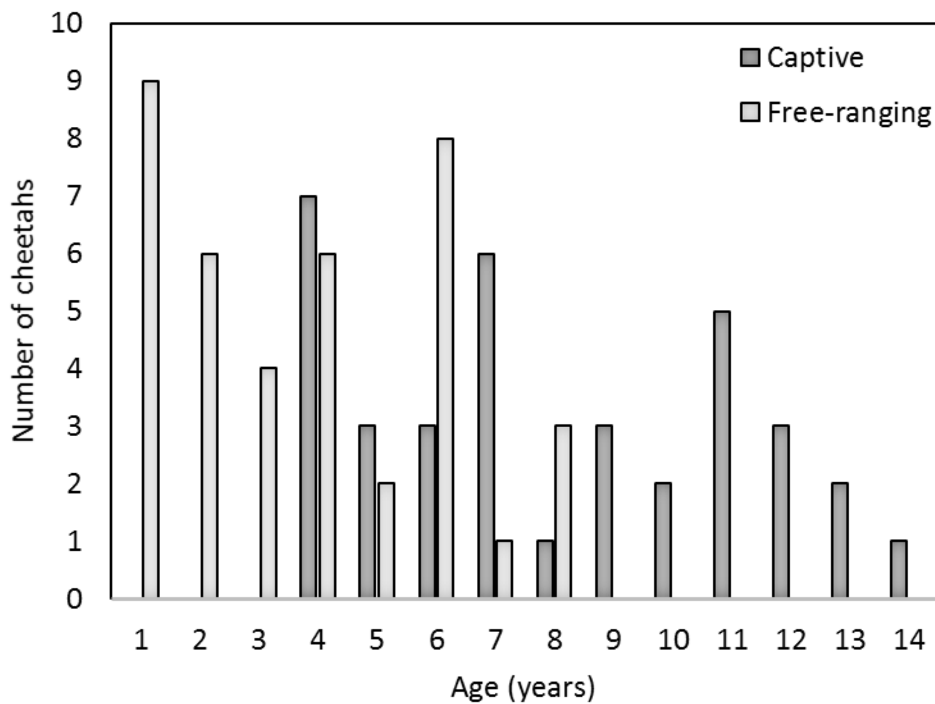

Figure 1: Histogram of ages for captive and free-ranging cheetahs

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4.2. Reference Intervals for Serum Analytes

Seven biochemistry analytes were measured in 66 healthy captive and free-ranging cheetahs and reference intervals were calculated for serum sodium, potassium, magnesium, chloride, urea, creatinine and osmolarity. Visual inspection of the data and the Anderson-Darling normality test showed that four of the seven variables were normally distributed (K, Mg, urea and creatinine). The distribution of data were non-Gaussian for sodium, chloride and osmolarity. Serum osmolarity was symmetrically distributed after Box-Cox transformation, however, sodium and chloride did not show Gaussian distribution or symmetry even after Box-Cox transformation. Both data sets were skewed to the left.

A single outlier was excluded in the calculation of the reference intervals for sodium and chloride, based on visual inspection of the data rather than using the Tukey or Dixon-Reed criteria, which only listed this value as suspect on the non-transformed data. The outlier for both sodium and chloride belonged to the same individual (P120) which had a very high sodium (175.6 mmol/l) and chloride (137.8 mmol/l) concentration (see Addendum A). Otherwise, no outliers were excluded from other variables.

The non-parametric method was used to calculate reference intervals for all variables, except for osmolarity where the robust method was used (Geffré et al. 2011). A non-parametric bootstrap method was used to calculate the 90% CIs of the reference limits, except for osmolarity where a parametric bootstrap method was used (Geffré et al. 2011). This is in accordance with the guidelines stipulated by the IFCC and CLSI, which are used by the American Society for Veterinary Clinical

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Pathology for their recommendations in generating reference intervals for veterinary species (Friedrichs et al. 2012).

The results of the statistical analyses and reference intervals are presented in Table 3. Values are compared with the ISIS values for all cheetahs in the ISIS database (mean, standard deviation, minimum and maximum value) (ISIS 2002).

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Table 3: Reference intervals (RI) for seven serum analytes and serum ISIS values of cheetahs

Serum Analyte	Study Population							Cheetah ISIS Values (ISIS 2002)				
	n	Mean	Median	SD	Minimum	Maximum	RI	n	Mean	SD	Minimum	Maximum
Sodium (mmol/l)	65	154	155	7	124	167	128 – 166	1066	157	4	128	175
Potassium (mmol/l)	66	4.5	4.5	0.4	3.8	5.3	3.9 – 5.2	1068	4.4	0.5	3.1	7.0
Magnesium (mmol/l)	66	1.0	1.0	0.1	0.8	1.2	0.8 – 1.2	26	1.0	0.1	0.8	1.5
Chloride (mmol/l)	65	120	122	7	96	130	97 – 130	1015	122	4	108	136
Urea (mmol/l)	66	13.9	13.3	4.0	8.1	26.2	8.2 – 25.1	1105	12.9	3.2	5.3	29.6
Creatinine (µmol/l)	66	175	172	44	85	303	88 – 288	839	212	80	53	716
Osmolarity (mOsmol/l)	20	328	328	15	281	363	293 – 356	20	322	16	298	355

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4.2.1. Comparison of Serum Analytes between Captive and Free-ranging Cheetahs

Common descriptive statistical analyses, separate reference intervals and *P*-values for the comparison of serum analytes (using the MWU test) between the captive and free-ranging cheetahs are presented in Table 4.

Partitioning criteria according to the ASVCP (ASVCP 2015) were applied to the captive and free-ranging cheetahs where separate reference intervals were determined for the serum analytes (Table 4), except for osmolarity due to the small sample size in each sub-group (n < 20) (ASVCP 2015). The robust method with Box-Cox transformation of the data was used to calculate reference intervals for the sub-group's serum analytes, except for urea of the captive population where the robust method with untransformed data was used. A parametric bootstrap method was used to calculate the 90% CIs of the reference limits.

There was a significant difference for serum sodium ($P = 0.02$) and urea ($P < 0.01$) between the captive and free-ranging groups. The median sodium concentration differed by 3 mmol/l between captive (154 ± 3 mmol/l) and free-ranging cheetahs (157 ± 10 mmol/l) (Table 4). The median urea concentration of captive cheetahs (7.4 – 22.9 mmol/l) was higher than that of free-ranging cheetahs (7.2 – 21.4 mmol/l) (Table 4 and Figure 2).

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Table 4. Statistics and RI for serum analytes for captive and free-ranging cheetahs, with *P*-values using the Mann-Whitney U test

Serum Analyte	Captive							Free-ranging (n = 36)							
	n	Mean	Median	SD	Min	Max	RI	n	Mean	Median	SD	Min	Max	RI	*P*-value
Sodium (mmol/l)	30	154	154	3	142	153	148 – 160	36	155	157	10	124	176	137 – 178	0.02
Potassium (mmol/l)	30	4.5	4.5	0.3	3.9	5.1	3.8 – 5.2	36	4.5	4.5	0.4	3.8	5.3	3.7 – 5.4	0.93
Magnesium (mmol/l)	30	1.0	1.0	0.1	0.9	1.2	0.9 – 1.2	36	1.0	1.0	0.1	0.8	1.2	0.7 – 1.2	0.67
Chloride (mmol/l)	30	122	122	3	111	126	116 – 129	36	119	122	9	96	138	103 – 140	0.50
Urea (mmol/l)	30	15.9	15.7	3.7	10.5	26.2	7.4 – 22.9	36	12.3	11.4	3.5	8.1	20.0	7.2 – 21.4	< 0.01
Creatinine (µmol/l)	30	184	183	39	111	273	114 – 276	36	169	163	47	85	303	89 – 283	0.11
Osmolarity (mOsmol/l)	12	327	327	5	318	334	-	8	330	330	24	281	363	-	-*

*No value could be calculated due to small sample size.

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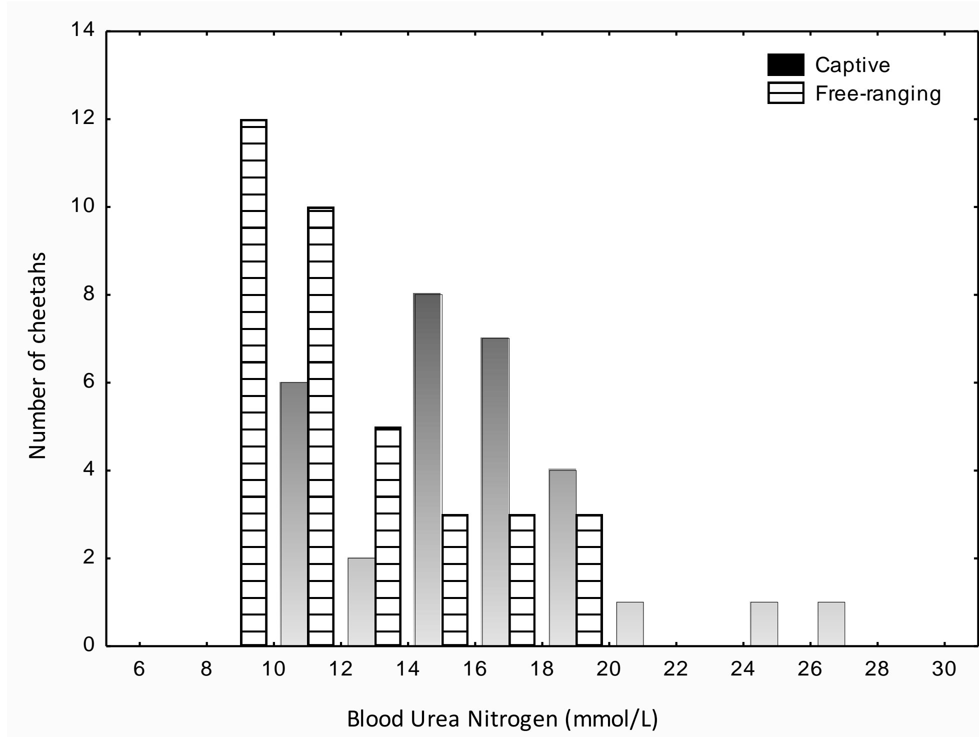

Figure 2: Histogram of serum urea concentration between captive and free-ranging cheetahs

4.2.2. The Effect of Age on Serum Analytes

Spearman's correlation co-efficients for serum analytes based on age are provided in Table 5. A correlation co-efficient of $r_s > 0.6$ and < -0.6 was regarded as a strong correlation (Weir n.d.).

Table 5: Spearman's correlation co-efficients for measured serum analytes associated with age

Serum Analyte	All cheetahs (n)	Captive (n)	Free-ranging (n)
Sodium	-0.07 (66)	-0.22 (30)	0.32 (36)
Potassium	-0.31 (66)	-0.46 (30)	-0.36 (36)
Magnesium	0.32 (66)	0.37 (30)	0.45 (36)
Chloride	0.31 (66)	0.22 (30)	0.42 (36)
Urea	0.32 (66)	0.32 (30)	-0.12 (36)
Creatinine	0.07 (66)	0.09 (30)	-0.20 (36)
Osmolarity	0.09 (20)	0.44 (12)	0.39 (8)

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There were no serum analytes which had a strong correlation with age, and therefore, no reference intervals according to age were calculated.

4.2.3. The Effect of Sex on Serum Analytes

The MWU test was used to statistically evaluate any differences in the serum analytes between male and female cheetahs (Table 6).

Table 6: MWU results for serum analytes between male and female cheetahs

Serum Analyte	All cheetahs (n)	Captive (n)	Free-ranging (n)
Sodium	0.64 (67)	0.75 (30)	0.90 (36)
Potassium	0.32 (67)	0.42 (30)	0.02 (36)
Magnesium	0.43 (67)	0.37 (30)	0.88 (36)
Chloride	0.41 (67)	0.41 (30)	0.80 (36)
Urea	0.04 (67)	0.55 (30)	0.12 (36)
Creatinine	0.37 (67)	0.16 (30)	0.78 (36)
Osmolarity	0.71 (20)	-	-

Serum urea was the only analyte which had a significant result of P = 0.04, where urea was higher in female cheetahs (15.6 ± 4.0 mmol/l) than male cheetahs (12.0 ± 3.9 mmol/l).

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4.2.4. The Effect of Haemolysis on Serum Analytes

The serum samples from the free-ranging cheetahs were significantly more haemolysed than those from the captive cheetahs (Chi-square test, $X^2 = 37.7$, degrees of freedom = 2, $P < 0.01$). From Table 7 it can be seen that 5.7% (2/35) of the captive cheetah samples had a haemolysis score of > 1+, compared to 65.8% (25/38) of the free-ranging cheetah samples that had the same haemolysis score. In addition, 51.4% (18/35) of captive cheetah samples had no haemolysis, whereas all of the free-ranging cheetah samples had some degree of haemolysis.

Table 7: Haemolysis scores of serum samples between captive and free - ranging cheetahs

Haemolysis Score	Captive	Free-ranging	Total
0	18	0	18
1+	15	13	28
> 1+	2	25	27
Total	35	38	73

Table 8 gives the means and standard deviations of the measured serum analytes based on haemolysis score. The serum analytes were unaffected by haemolysis except for urea, which was lower in serum samples with haemolysis scores of 1+ and > 1+ (Kruskal-Wallis test, $P = 0.02$ for both), compared to serum samples with no haemolysis.

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Table 8: Mean ± SD of serum analytes of all cheetahs, based on haemolysis score

Serum Analyte	0		1+		>1+	
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
Sodium (mmol/l)	14	155 ± 1.6	26	154 ± 7.1	26	155 ± 10.2
Potassium (mmol/l)	14	4.4 ± 0.3	26	4.6 ± 0.4	26	4.5 ± 0.4
Magnesium (mmol/l)	14	1.0 ± 0.1	26	1.0 ± 0.1	26	1.0 ± 0.1
Chloride (mmol/l)	14	123 ± 2.5	26	121 ± 6.7	26	119 ± 8.6
Urea (mmol/l)	14	16.9 ± 4.3	26	13.1 ± 3.6	26	13.1 ± 3.7
Creatinine (µmol/l)	14	190 ± 38.1	26	167 ± 39.2	26	176 ± 50.6
Osmolarity (mOsmol/l)	7	326 ± 5.4	8	321 ± 17.2	5	340 ± 15.9

4.3. Urine Analytes

The means, medians, standard deviations, and minimum and maximum values for the measured urine analytes of the study population are presented in Table 9.

There were some urine analytes where the median values were found to be similar between the captive and free-ranging cheetahs. These included uric acid, urine osmolarity and urine SG. However, there was a large difference between some of the captive and free-ranging cheetah's urine analytes such as urine sodium, urine potassium, urine magnesium, urine chloride, urine protein, urine creatinine and UPC. Captive cheetahs were found to have lower levels of urine sodium, potassium, magnesium, urine protein and UPC than free-ranging cheetahs. Urine chloride and urine creatinine were higher in the captive cheetahs compared to the free-ranging cheetahs.

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Table 9: SG-corrected urine analytes in captive and free-ranging cheetahs

Urine Analyte	Captive						Free-ranging					
	n	Mean	SD	Median	Min Value	Max Value	n	Mean	SD	Median	Min Value	Max Value
Sodium (mmol/l)*	10	35.16	20.85	33.13	6.03	85.08	3	45.90	6.42	44.23	40.48	53
Potassium (mmol/l)	28	91.34	40.08	83.42	31.71	185.13	5	113.08	65.61	130.91	41.97	175.96
Magnesium (mmol/l)	28	3.63	1.53	3.45	1.08	5.84	5	5.53	2.63	5.42	2.53	9.60
Chloride (mmol/l)	28	77.97	52.40	62.36	26.77	238.08	5	47.37	17.20	45.68	28.95	69.10
Uric Acid (mmol/l)	28	0.42	0.23	0.37	0.17	1.38	5	0.79	0.53	0.50	0.38	1.65
Urine Protein (g/l)	28	0.74	0.67	0.41	0.15	2.26	5	1.40	0.87	1.57	0.34	2.51
Creatinine (mmol/l)	31	48.24	21.09	50.05	21.02	105.69	6	35.12	24.87	21.57	14.52	68.04
Aldosterone (pmol/l)	17	18.61	31.35	7.00	3.3	121.99	0	-	-	-	-	-
Urine SG	31	1.050	0.01	1.054	1.030	1.080	6	1.051	0.02	1.055	1.026	1.080
Osmolarity (mOsmol/l)	23	2248.8	400.7	2198	1549	2956	2	2053.5	1144.8	2053.5	1244	2863
UPC	28	0.158	0.141	0.136	0.021	0.660	5	0.586	0.508	0.620	0.045	1.109

*Only urine sodium values greater than zero were used.

A comparison of urine sodium measurements between captive and free-ranging cheetahs is depicted in Table 10, demonstrating that 64.3% (18/28) of captive cheetahs had no or undetectable amounts of sodium in their urine, whereas 40% (2/5) of free-ranging cheetahs had undetectable amounts of urine sodium. It is also important to note that 60.6% (20/33) of the total study population had no or trace amounts of urine sodium present, with 90% (18/20) of those cheetahs being captive cheetahs.

Table 10: Comparison of SG-corrected urine sodium levels between captive and free-ranging cheetahs

Level of urine sodium	Captive (n)	Free-ranging (n)	Total
> 0 mmol/l	10	3	13
Zero / Undetectable amounts	18	2	20
Total	28	5	33

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4.3.1. The Effect of Age on Urine Analytes

Spearman's correlation co-efficients for urine analytes associated with age are given in Table 11. There are no values for the free-ranging cheetahs due to the very small sample size. A correlation co-efficient of $r_s > 0.6$ and < -0.6 is regarded as a strong correlation (Weir n.d.).

Table 11: Spearman's correlation co-efficients for SG-corrected urine analytes associated with age

Urine Analyte	All cheetahs (n)	Captive (n)	Free-ranging (n)
Sodium*	-0.55 (13)	-0.40 (10)	- (3)
Potassium	0.23 (33)	0.15 (28)	- (5)
Magnesium	-0.10 (33)	0.12 (28)	- (5)
Chloride	-0.20 (33)	-0.22 (28)	- (5)
Uric acid	-0.01 (33)	0.15 (28)	- (5)
Creatinine	0.24 (37)	0.02 (31)	- (6)
Urine protein	-0.09 (33)	0.12 (28)	- (5)
Aldosterone	0.40 (17)	0.40 (17)	- (0)
Urine SG	0.36 (37)	0.26 (31)	- (6)
Osmolarity	0.36 (25)	0.22 (23)	- (2)
UPC	-0.08 (33)	0.20 (28)	- (5)

*Only urine sodium values greater than zero were used.

There were no urine analytes which showed a strong correlation with age in the study. Urine aldosterone had a moderate positive correlation with age ($r_s = 0.40$) (Table 11). However, by observing Figure 3, it can be seen that if the two very high outliers were to be removed from the scatter plot, urine aldosterone would show no correlation with age.

Urine sodium showed a moderate negative correlation with age ($r_s = -0.55$) (Table 11 and Figure 4). Urine potassium, urine SG and osmolarity had weak positive correlations and urine magnesium, chloride, uric acid, urine protein and UPC had weak negative correlations with age (Table 11).

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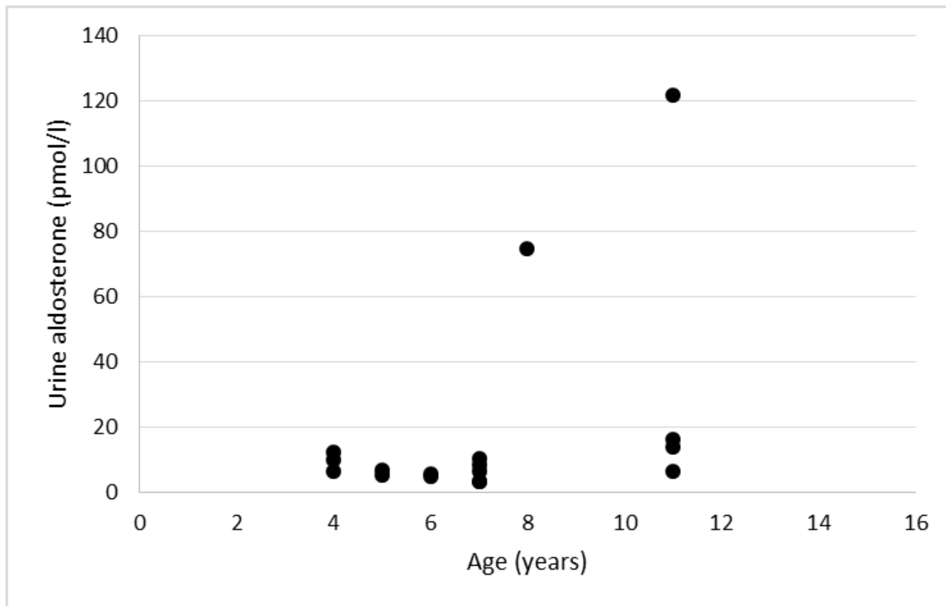

Figure 3: Scatter plot of SG-corrected urine aldosterone based on age

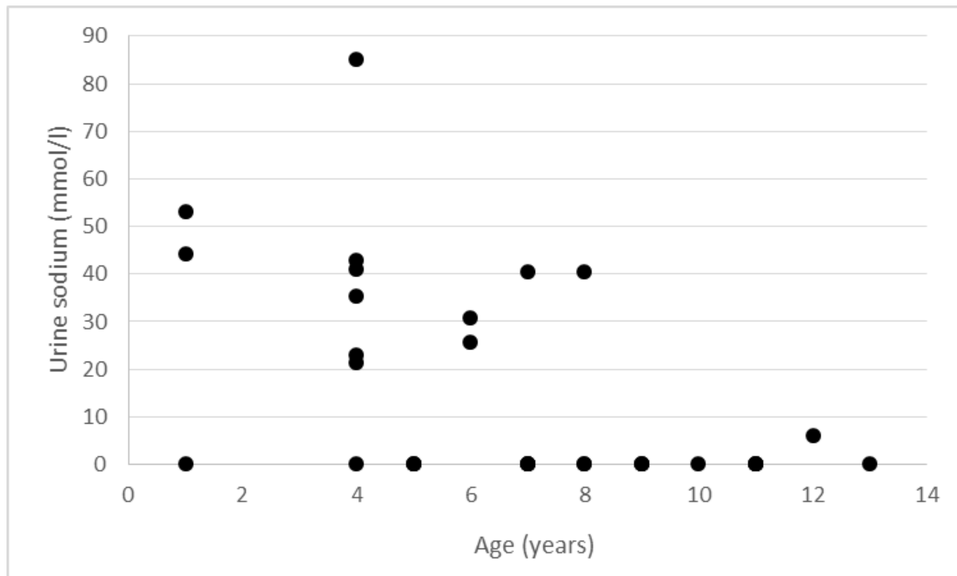

Figure 4: Scatter plot of SG-corrected urine sodium based on age

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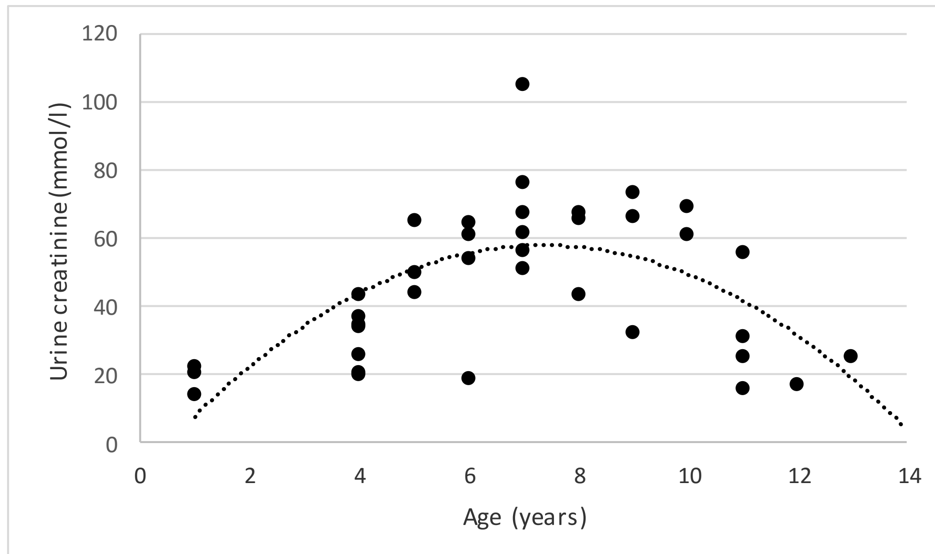

Figure 5. Scatter plot of SG-corrected urine creatinine based on age (r_s = 0.24)

The relationship between urine creatinine and age was not monotonic (Weir n.d.) and follows a quadratic relationship as can be observed in Figure 5, which demonstrates that urine creatinine is lower in young and aged cheetahs.

4.3.2. The Effect of Sex on Urine Analytes

There were no urine analytes that differ significantly between male and female cheetahs of the study population.

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4.4. Correlations between Variables

Spearman correlations were performed between the various serum and urine analytes that were measured. The full correlation analysis can be viewed in Addendum B. Table 12 provides a summary of Spearman correlations that had a correlation co-efficient of $r_s > 0.6$ or < -0.6, and $P < 0.05$.

Table 12: Spearman's correlation co-efficients between various serum and SG-corrected urine analytes of all cheetahs (correlation coefficients of $r_s > 0.6$ or < -0.6, $P < 0.05$)

Analyte	Correlated Analyte	Spearman's Correlation Coefficient (n)
Serum Sodium	Serum chloride	0.69 (66)
Serum Creatinine	Urine creatinine	0.64 (28)
Urine Aldosterone	Urine creatinine	-0.62 (17)
Urine Osmolarity	Urine SG	0.93 (25)
	Urine sodium	-0.98 (8)
UPC	Urine protein	0.86 (33)

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CHAPTER 5

Discussion

There was a significant difference in the study population with regards to sex (Chi-Square test, P = 0.0499), age (MWU test, P < 0.01) and haemolysis score (MWU test, $P < 0.01$).

There were significantly more male cheetahs (67%) compared to female cheetahs (33%) in the study population (Table 1). This was because of the high proportion of male cheetahs in the free-ranging population (76.9%) due to the fact that the box traps used to capture the free-ranging cheetahs were placed at cheetah marking trees which are more often frequented by males than females. Marker et al. (2003) performed a study on the demographics of Namibian free-ranging cheetahs and found that there was a strong bias towards capturing adult males, with 2.9 males captured for every adult female. This is a significant deviation from a 1:1 sex ratio. Marker et al. (2003) attributed the skewed sex ratio to a sampling bias rather than a true indication of population structure in the wild.

The sex of the cheetahs had no significant effect on the serum analytes measured in this study except for serum urea (MWU test, P = 0.04) (Table 6), where urea was higher in female (15.6 ± 4.0 mmol/l) than male cheetahs (12.0 ± 3.9 mmol/l). However, there was no statistical significance between male and female cheetahs in their respective sub-groups for urea concentrations (MWU test, Captive: P = 0.55, Free-ranging: P = 0.12) (Table 6). Since the majority of female cheetahs in this study are captive (64%) (Table 2), and captive cheetahs had higher urea concentrations

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(15.7 ± 3.7 mmol/l) than free-ranging cheetahs (11.4 ± 3.5 mmol/l) (Table 4), a true sex-related difference in urea concentration in the study population is unlikely. Thus, the higher urea levels in female cheetahs is most likely due to the sampling bias as there were significantly more captive females than free-ranging females.

The age demographics of the study population also differed between the captive and free-ranging cheetahs (Figure 1). The captive cheetahs were significantly older than the free-ranging cheetahs (MWU Test, $P < 0.01$), where the median age of the captive cheetahs was seven years (Inter-quartile range: 5 – 11 years), compared to four years (Inter-quartile range: 2 – 6 years) for the free-ranging cheetahs. There were no captive cheetahs below four years of age (Figure 1). The AfriCat Foundation does not breed any captive carnivores, and almost all of the cheetahs were orphaned cubs which have subsequently been in captivity for a number of years already, hence the absence of young cheetahs. The highest peak of mortality in free-ranging cheetahs is between five and six years of age, with the maximum age recorded for a free-ranging Namibian cheetah being 12 years (Marker et al. 2003). This is consistent with the findings in this study where there were a small number of free-ranging cheetahs over six years of age (n = 4). The age of the cheetahs had no significant effect on the serum analytes measured in this study (Table 5).

As a result of the captive and free-ranging cheetah samples being collected by different researchers, the consistent quality of the samples that were analysed cannot be guaranteed. This was quite evident in the qualitative haemolysis scores of the serum samples between captive and free-ranging cheetahs, where the free-ranging cheetah samples had significantly more haemolysis than the captive cheetah samples (Chi-square test, $P < 0.01$) (Table 7). Factors that have been suggested to cause increased sample haemolysis include: the use of a vacutainer system,

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prolonged time between sample collection and centrifugation and operator-related factors such as operator experience (Grant 2003; Heiligers-Duckers et al. 2013; Ong, Chan & Lim 2009), all of which apply to the free-ranging cheetah samples and are the most likely causes for the difference observed in sample haemolysis between captive and free-ranging cheetahs.

From the results under Section 4.2.4 and Table 8 it can be seen that haemolysis had no significant effect on the serum analytes measured, except for urea which was lower in serum samples with haemolysis scores of 1+ (13.1 ± 3.6 mmol/l) and > 1+ (13.1 ± 3.7 mmol/l), compared to serum samples with a haemolysis score of 0 (16.9 ± 4.3 mmol/l) (Kruskal-Wallis test, $P = 0.02$ for both). This result may be attributed to the fact that all of the serum samples (n = 18) with a haemolysis score of 0 were samples from captive cheetahs (Table 7). It has been shown in Table 4 that captive cheetahs had higher urea levels than free-ranging cheetahs. Therefore, the observation of haemolysis causing a lowered urea level is due to a sampling bias.

Serum potassium is the electrolyte most affected by haemolysis in humans (Frank et al. 1978) and other species such as horses, pigs, some ruminants and rare dog breeds (Kaneko, Harvey & Bruss 2008). When serum potassium determinations are performed using ion-selective electrodes, a direct correlation is observed between the degree of haemolysis and serum potassium measurements due to the high intracellular concentration of potassium in the erythrocytes of the above-mentioned species (Kaneko, Harvey & Bruss 2008; Koseoglu et al. 2011). In contrast, the erythrocytes of cats, ferrets and most dogs have intracellular potassium concentrations similar to that of plasma (Kaneko, Harvey & Bruss 2008), and therefore, haemolysis does not affect serum potassium measurements (using ISE indirect method) in those species. Based on the result in Table 8 where haemolysis

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had no significant effect on serum potassium in cheetahs, it is likely that cheetah erythrocytes also have intracellular potassium concentrations similar to plasma.

This study provides reference intervals for serum electrolytes (sodium, potassium, magnesium and chloride), serum urea, creatinine and osmolarity (Table 3). No separate reference intervals were calculated for the study population as well as the captive and free-ranging sub-groups according to age, sex and haemolysis score in this study as these factors had no significant influence on the serum analytes measured.

There were no explicit differences when comparing the reference intervals of the study population with the cheetah ISIS values that have been recorded (Table 3). The mean cheetah ISIS values for the measured serum analytes all fell within the reference intervals that were calculated, and were very similar to the mean values of the study population, except for creatinine (212 µmol/l) which was 1.2 times higher than the study population (175 µmol/l). The minimum ISIS values fell outside the respective reference interval, except for sodium, chloride and osmolarity. The maximum ISIS values were all above the reference intervals for the serum analytes in this study, except for osmolarity. The interpretation of these findings is complicated by the fact that the ISIS values are unpublished data consisting of captive animals of unknown health status in zoological institutions (ISIS 2002). Multiple samples may also be from the same individual and analytical methods are not described (ISIS 2002). Furthermore, normality of these data are not reported, thus it is unclear if the means and standard deviations can be used to establish reference intervals (Maas, Keet & Nielen 2013).

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Except for serum osmolarity, separate reference intervals for the captive and free-ranging sub-groups were calculated according to ASVCP guidelines (ASVCP 2015) (Table 4). As there were less than 40 samples for each sub-group, the uncertainty and imprecision of the reference limits may be very high (Geffré et al. 2009). Nevertheless, there were large differences in the reference intervals for serum sodium, chloride and creatinine; where these analytes had reference intervals that were wider in the free-ranging cheetahs.

When statistically comparing the serum analytes between captive and free-ranging cheetahs in this study, it was observed that there was a significant difference for serum sodium (MWU test, $P = 0.02$), and urea (MWU test, $P < 0.01$) (Table 4).

The captive cheetahs have a lower median sodium value (154 mmol/l) than free-ranging cheetahs (157 mmol/l). The captive cheetahs are not, however, hyponatraemic as their sodium reference interval of 148 – 160 mmol/l (Table 4) is very similar to that of domestic cats (Table 13: 144 – 156 mmol/l). The reasoning for the lower sodium level could be linked to total body sodium depletion which is explained in further detail below on pages 49 – 50.

The difference in urea concentrations between captive and free-ranging cheetahs was weakly associated with the difference in ages between the two sub-groups according to the Spearman's correlation co-efficient ($r_s = 0.32$, $P < 0.01$) (Table 5), thus ruling out age as a significant factor. The MWU test showed a significant difference between male and female urea concentrations ($P = 0.04$), where the urea concentrations were higher in females than males (Table 6). This finding was also noted with the mean urea ISIS value being higher in female cheetahs (12.5 mmol/l in males and 13.2 mmol/l in females) (ISIS 2002). There was no significant difference

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between male and female cheetahs in their respective sub-groups (Table 6), and with the majority of female cheetahs being captive (Table 2), a sampling bias rather than a sex-related difference may explain the higher urea level in female cheetahs.

Causes for increases in urea concentrations are divided into three categories: pre-renal, renal and post-renal (DiBartola 2010). Pre-renal causes for elevated urea are increased protein catabolism (e.g. small bowel haemorrhage and necrosis, starvation, prolonged exercise, fever and corticosteroids), decreased renal perfusion with reduced glomerular filtration rate (shock, dehydration and cardiovascular disease) and high protein diets (Backlund et al. 2011; DiBartola 2010).

The cheetahs in captivity are fed a diet of 1.0 – 1.5 kg donkey meat on a daily basis, compared to their free-ranging counterparts that consume the muscle meat and viscera of small antelope, rodents and birds, and seldom eat daily (Bechert et al. 2002). The protein-to-fat ratio of supplemented meat diets (horse, beef and deer) commonly fed to captive cheetahs is 6.6:1, whereas whole prey ratios are only 3:1 (Bechert et al. 2002). Therefore, it can be seen that the protein content of captive cheetah’s diets is more than double that of free-ranging cheetahs. This high frequency feeding of large portions of high protein meat to captive cheetahs may result in elevated urea levels in comparison to free-ranging cheetahs. In a study by Caro et al. (1987) it was also found that captive cheetahs had higher urea levels than free-ranging cheetahs. This finding was attributed to a higher total food intake where it was found that free-ranging cheetahs were consuming about 75% of the captive diet (Caro et al. 1987). The cheetahs in captivity also have free access to water at all times, therefore, ruling out dehydration as a possible cause of elevated urea. Therefore, the feeding of a high protein diet to cheetahs in captivity can result in higher urea levels in these animals, compared to their free-ranging counterparts.

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The high protein diet could, however, mask elevated urea levels due to gastritis (small bowel haemorrhage) and/or renal disease (renal azotaemia) in captive cheetahs (Chew, DiBartola & Schenck 2011; DiBartola 2010). Lymphoplasmacytic gastritis and renal disease are two of the most prevalent diseases of captive cheetahs, and are rare in free-ranging cheetahs (Munson et al. 2005). Bolton and Munson (1999) stated that the daily feeding of high protein diets to captive cheetahs may influence the development of glomerulosclerosis. Glomerulosclerosis is one of the two main lesions seen in captive cheetahs with renal disease, the other being renal amyloidosis (Bolton & Munson 1999). However, a concurrent and proportional increase in creatinine would be expected in cases of renal azotaemia, which was not the case in this study. Therefore, the captive cheetahs in this study may also be suffering from lymphoplasmacytic gastritis, resulting in pre-renal elevations of urea.

There was no evidence for post-renal causes of elevated urea in the captive cheetahs as none were suffering from urinary tract obstruction. In conclusion, pre-renal causes (high protein diet and/or lymphoplasmacytic gastritis) are responsible for elevated urea concentrations in captive cheetahs in this study.

When comparing the reference intervals of the serum analytes in this study to those of domestic cats (Table 13), it can be seen that the serum electrolytes and osmolarity are very similar, whereas, the serum urea and creatinine reference intervals are much higher in cheetahs, compared to domestic cats. The reference intervals for captive and free-ranging cheetahs for urea (7.4 – 22.9 mmol/l and 7.2 – 21.4 mmol/l, respectively) and creatinine (114 – 276 μmol/l and 89 –283 μmol/l, respectively) (Table 4) are also higher than those for domestic cats.

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Table 13: Reference intervals for serum analytes in cheetahs in this study and domestic cats

Serum Analyte	Cheetahs	Domestic cats*
Sodium	128 – 166 mmol/l	144 – 156 mmol/l
Potassium	3.9 – 5.2 mmol/l	3.8 – 5.1 mmol/l
Magnesium	0.8 – 1.2 mmol/l	0.7 – 1.2 mmol/l
Chloride	97 – 130 mmol/l	113 – 124 mmol/l
Urea	8.2 – 25.1 mmol/l	4.1 – 12.9 mmol/l
Creatinine	88 – 288 µmol/l	57 – 138 µmol/l
Osmolarity	293 – 356 mOsmol/l	280 – 305 mOsmol/l

*Reference intervals used by the Veterinary Diagnostic Laboratory, Onderstepoort, Pretoria, South Africa (December 2014).

The higher serum urea and creatinine reference intervals in cheetahs could be dietary related. The protein-to-fat ratio of captive and free-ranging cheetah diets is 6.6:1 and 3:1 respectively (Bechert et al. 2002). Commercial adult domestic cat food has a protein-to-fat ratio of 1.5 – 1.7:1 (Eukanuba 2016). Thus, the protein content of cheetahs' diets (captive and free-ranging) is higher than that of domestic cats'. High protein diets can result in higher serum urea and creatinine levels (Bechert et al. 2002, Miller et al. 1999). Other possible explanations for higher creatinine values in cheetahs may include: species variability, a response to acute myositis resulting from the intramuscular ketamine injection (although this explanation is unlikely, given the timeliness of collection), and/or greater muscle mass (i.e. greater energy stores) in cheetahs than in domestic cats (Miller et al. 1999).

Furthermore, it has been shown that captive cheetahs fed a commercial cat food diet took longer to develop moderate-severe gastritis and renal disease than those fed a supplemented meat-based diet (Lane et al. 2012). This suggests that a commercial

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cat food diet, which is lower in protein, may be more beneficial in slowing down the onset and progression of gastritis and renal disease in captive cheetahs.

Urine analytes, except for urine osmolarity, were adjusted and corrected relative to urine SG in this study. It has become common practice to correct analytical values determined from spot samples (Alessio et al. 1985). The main objective of adjustment is to reduce variations due to inconstant dilution of spot samples, which may fluctuate considerably in the same subject in the course of the same day (Alessio et al. 1985). Methods for correction of hormone/metabolite concentrations in urine have been used by anti-doping laboratories, pain monitoring programmes and environmental monitoring programmes (Cone et al. 2009). Two correction methods are frequently used, based on SG and urine creatinine (Berlin et al. 1985). In humans, SG and urine creatinine values have been shown to be significantly correlated.

Urine creatinine is widely used to standardise the concentration of urine samples because theoretically it is metabolised and excreted in urine at a constant rate (Anestis et al. 2009; Muscat, Liu & Richie 2011). However, there are a few problems with this method of adjustment as it has been found that there is considerable intra- and inter-individual variation in creatinine excretion rates (Alessio et al. 1985; Berlin et al. 1985; Miller et al. 2004). Lean body mass, diet, diurnal variation and muscle activity causes variation within individuals (Alessio et al. 1985; Anestis et al. 2009; Miller et al. 2004; Muscat, Liu & Richie 2011; White et al. 2010). Age, sex and disease status results in variation between individuals (Alessio et al. 1985; Anestis et al. 2009; Miller et al. 2004; White et al. 2010). Measuring urine creatinine adds time and expense to hormone/metabolite assessment (White et al. 2010). Furthermore, creatinine is unstable in storage at -80 °C and sensitive to degradation as a result of

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freeze/thaw cycles (Anestis et al. 2009; White et al. 2010). The measurement of creatinine may require an additional freeze/thaw cycle which may affect the accurate measurement of creatinine and/or the hormone/metabolite of interest (White et al. 2010).

Specific gravity is the ratio of the density of urine to the density of distilled water, and a measure of total soluble solids (Muscat et al. 2011; White et al. 2010). The most abundant molecules in urine are urea, electrolytes, creatinine and other metabolic waste products (Muscat et al. 2011). Urine SG may vary by disease states that increase urinary glucose and protein quantities, such as diabetes mellitus and nephrotic syndrome (Cone et al. 2009; Miller et al. 2004; Muscat et al. 2010). However, urine SG can be measured accurately, quickly and inexpensively using a handheld refractometer (Anestis et al. 2009). It can be measured at the time and location of initial sample collection, avoiding unnecessary freeze/thaw events (White et al. 2010). Small volumes are sufficient to measure urine SG making it useful in cases where samples are small (White et al. 2010).

The measurement of urine electrolytes (sodium, potassium and chloride) can be very informative and useful in the diagnostic evaluation of volume status, hyponatraemia, acute kidney injury, metabolic alkalosis, hypokalaemia and urine anion gap (Carmody 2011; Reddi 2014). A spot urine sample is generally adequate for determination of these electrolytes and should be interpreted in the context of the patient and the clinical situation (Reddi 2014). Urine creatinine is determined to calculate the fractional excretion of sodium, potassium and other electrolytes (Reddi 2014). Urine osmolarity is useful in the differential diagnosis of hyponatraemia, polyuria and acute kidney injury (Reddi 2014).

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The basic statistics for the urine analytes in Table 9 can be used by practitioners, but should however, be used with caution due to the fact that reference intervals could not be calculated as a result of small sample size (particularly the free-ranging cheetahs), and there are many intrinsic (species-related, age, sex) and extrinsic (habitat/environment, diet) factors influencing the excretion of the urine analytes that were measured in this study. Furthermore, there are no published data on reference intervals for the urine analytes measured, making evaluation and comparison with published data difficult.

The age and sex of the cheetahs in this study had no significant effect on the urine analytes. Any differences in urine analytes between captive and free-ranging cheetahs could not be statistically quantified due to the small sample size of the free-ranging cheetahs. The discussion will, therefore, be limited to the captive cheetahs only. Urine sodium, aldosterone and creatinine will be discussed in more detail.

Sodium is the body's major extracellular cation, and plays a major physiologic role in maintaining extracellular volume (Louden 2012). Sodium and water are both freely filtered into Bowman's space and 99% of both are reabsorbed and none is secreted. Two thirds of this reabsorption occurs in the proximal tubule, but the major hormonal control is exerted on the distal convoluted tubules and collecting ducts. Sodium reabsorption is an active process and water reabsorption is by diffusion, dependent on sodium reabsorption. The major control of active tubular sodium reabsorption is by aldosterone, which stimulates sodium reabsorption in the collecting ducts in exchange for potassium which is excreted in the urine (Louden 2012). The renin-angiotensin-aldosterone system (RAAS) and extracellular potassium concentration are the two major controllers of aldosterone secretion, and adrenocorticotrophic hormone (ACTH) to a lesser extent (Louden 2012). The RAAS is activated in

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response to a decrease in circulating blood volume (hypovolemia) and renal perfusion (Buranakarl, Mathur & Brown 2004; Ibrahim, Rosenberg & Hostetter 1997). This leads to a decrease in the delivery of sodium and chloride to the macula densa of the distal tubules and results in renin secretion (Buranakarl, Mathur & Brown 2004; Ibrahim, Rosenberg & Hostetter 1997). Renin cleaves angiotensinogen (produced in the liver) to angiotensin I which is hydrolysed in the lungs by angiotensin-converting enzyme into angiotensin II (Buranakarl, Mathur & Brown 2004; Ibrahim, Rosenberg & Hostetter 1997). Angiotensin II stimulates the secretion of aldosterone from the zona glomerulosa in the adrenal gland (Syme et al. 2007). Typically, sodium and chloride are reabsorbed together throughout the nephron and accordingly, the urine sodium and chloride levels are approximately equal (Carmody 2011).

Urine sodium is used mainly to assess a patient's volume status, and is particularly useful in the evaluation of hyponatraemia and acute renal failure (Carmody 2011). When the effective circulating volume is decreased, the kidney retains sodium in an effort to maintain circulating volume, and the urine sodium level is low (Carmody 2011). In this study, captive cheetahs showed an increased prevalence of no/undetectable amounts of sodium in the urine. Table 10 showed that 60.6% of cheetahs had urine sodium levels below the level of detection, and 90% of those cheetahs were captive cheetahs. In addition, Table 11 and Figure 4 showed that there was a moderate negative correlation between urine sodium and age.

Changes in water excretion by the kidney can result in changes in urine sodium concentration (Reddi 2011). To correct for water reabsorption, the renal handling of sodium can be evaluated directly by calculating the fractional excretion (FE) of sodium (Reddi 2011). The fractional excretion of sodium is defined as the ratio of urine (U_{Na}) to plasma sodium (P_{Na}) divided by the ratio of urine (U_{Cr}) to plasma

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creatinine (P_{Cr}), multiplied by 100 (Reddi 2011). This is demonstrated by the following formula (Carmody 2011, Reddi 2011):

$$FE_{Na} (\%) = (U_{Na} \times P_{Cr}) / (P_{Na} \times U_{Cr}) \times 100$$

Appropriate sodium conservation results in a $FE_{Na} < 1\%$ compared to patients with acute tubular necrosis whose FE_{Na} is generally high (> 2%) (Carmody 2011, Reddi 2011). The FE_{Na} in cheetahs with no or low levels of urine sodium is zero or very close to zero. Sodium conservation and low fractional excretion of sodium are avid in animals with pre-renal azotaemia and volume depletion (Carmody 2011, DiBartola 2010, Reddi 2011). Pre-renal azotaemia (more specifically, the high protein diet), which was also implicated in the finding of higher serum urea levels in captive cheetahs, may thus explain the high proportion of captive cheetahs with low fractional excretion of sodium.

A study by Kang et al. (2012) demonstrated that mean spot urine sodium is a simple and effective method that can be used to monitor sodium intake in human patients with chronic kidney disease (CKD). It was found that there is a strong correlation between mean spot urine sodium and with sodium intake by recall (Kang et al. 2012). Therefore, a measurement of zero or trace amounts of urine sodium, can be closely correlated to a low sodium intake.

There are no published data on the minimum daily sodium requirements of young, adult and geriatric cheetahs. Adult domestic cats have a minimum daily sodium requirement of 800 mg Na/kg diet (as fed)/day (Yu & Morris 1999). The minimum daily sodium requirement of domestic kittens is double that of adult domestic cats, i.e. 1.6 g Na/kg diet (Yu & Morris 1997). The sodium content of bovine and horse meat fed to cheetahs in captivity is 381 – 510 mg/kg WB (Williams 2007) and 295 –

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380 mg/kg WB (Lee et al. 2007) respectively. If the minimum daily sodium requirement of domestic cats (both adult and young) is extrapolated to cheetahs, it can be seen that cheetahs in captivity are fed a diet that is potentially sodium deficient.

Low sodium nutrition is known to result in the depletion of total body (tissue) sodium in humans (Blank et al. 2012). Total body sodium depletion is not immediately detectable by reduced plasma sodium concentrations (Blank et al. 2012). Plasma sodium levels may remain in low but normal ranges due to the complex regulation via the RAAS (Blank et al. 2012). This may explain why the captive cheetahs in this study have lower (but normal) serum sodium levels than free-ranging cheetahs.

Therefore, the diet of the captive cheetahs in this study which is high in protein and low in sodium can explain the higher serum urea levels, lower serum sodium levels and low fractional excretion of sodium.

There was a weak negative correlation between serum sodium levels and age in captive cheetahs, compared to a moderate positive correlation in free-ranging cheetahs (Table 5). This could also indicate that the older captive cheetahs are suffering from total body sodium depletion (Blank et al. 2012) due to a possible increased dietary sodium requirement. It was observed that urine sodium levels decreased with age (Table 11 and Figure 4), and the geriatric cheetahs received the same diet as the juvenile, sub-adult and adult cheetahs in captivity, and therefore should theoretically be consuming the same amount of sodium, and excreting similar amounts in the urine. Other potential causes for sodium retention could be volume depletion (hypovolemia), pre-renal azotaemia (high protein diets), oedematous

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disorders (cirrhosis, congestive heart failure, and nephrotic syndrome), water intoxication and hyperadrenocorticism (Carmody 2011).

There are no published data on urine aldosterone measurements in cheetahs prior to this study. Therefore, normal reference intervals are not known. Urine aldosterone was significantly elevated in two cheetahs (Figure 3). These two cheetahs were captive cheetahs and both were over eight years of age. Both also had undetectable amounts of urine sodium. Spot urine aldosterone measurements are a commonly employed confirmatory test to detect hyperaldosteronism in humans (Wu et al. 2013). This may potentially indicate primary or secondary hyperaldosteronism in these individuals. Primary hyperaldosteronism in the domestic cat has been previously underdiagnosed and is becoming a more important cause of hypertension in domestic felines (Djajadiningrat-Laanen, Galac & Kooistra 2011). Tumours of the adrenal cortex resulting in excessive aldosterone secretion (and low plasma renin activity) have been described in domestic cats, and result in systemic hypertension and hypokalaemia. Histopathological lesions identified include hyaline arteriolar sclerosis, glomerulosclerosis, tubular atrophy and interstitial fibrosis (Javadi et al. 2005). Secondary hyperaldosteronism arises as a result of chronic renin activation and subsequent sustained RAAS activation caused by cardiovascular failure, renal failure or severe hepatocellular dysfunction (Andrew, Harvey & Tasker 2005). ACTH and hyperkalaemia are also responsible for aldosterone secretion (Briet 2014; Jepson, Syme & Elliot 2014). An article by Syme et al. (2007) demonstrated that the measurement of feline urine aldosterone has limited or no utility in investigating the dysregulation of the RAAS in the pathophysiology of feline hypertension. Further work is, therefore, required to determine the efficacy of spot urine aldosterone measurements in confirming hyperaldosteronism in felines, and to identify

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aldosterone metabolites and elucidate their major routes of excretion. The two cheetahs mentioned above with excessively high urine aldosterone may be suffering from some form of hyperaldosteronism. However, further investigation would need to be carried out to determine this. If the two outliers with high urine aldosterone levels are removed from the graph in Figure 3, the general trend of the graph will change to one that shows no correlation between age and urine aldosterone levels.

As mentioned previously, urine creatinine is used to standardise the concentration of urine samples (Anestis et al. 2009; Muscat et al. 2011). It is also used to calculate the fractional excretion of sodium, potassium and other electrolytes, as well as UPC because creatinine is metabolised and excreted at a constant rate (Reddi 2014). However, the excretion of creatinine in cheetahs in this study was not constant and varied with age (Figure 5). Creatinine excretion was lower in young and aged cheetahs, and higher in mature cheetahs. Therefore, these results raise doubts as to the validity of urine creatinine as an analyte that can be used for adjustment purposes and in the calculation of fractional excretion of electrolytes and UPC in cheetahs.

There are various factors which are mentioned above that cause both intra- and inter-individual variation in creatinine excretion. The effects of age as well as lean body mass on creatinine excretion may be playing a role in the results observed in this study. Chizzotti et al. (2008) showed that creatinine excretion decreased linearly with body weight in Holstein cattle (i.e. different stages of maturity). This behaviour was stated to be associated with variations in the proportion of muscle tissue: body weight ratio, with older animals losing muscle mass and, therefore, excreting less creatinine (Chizzotti et al. 2008). This only partially explains the decrease in creatinine excretion in older cheetahs as seen in Figure 5 as lean body mass on its

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own seems to be poorly correlated with urine creatinine excretion in cheetahs. The older cheetahs show a two to five fold decline in urine creatinine excretion (15 – 30 mmol/l in older cheetahs, compared to 60 – 80 mmol/l in adult cheetahs) (Figure 5), yet it is very unlikely that these animals experience a similar decline in lean body mass. The lower creatinine excretion in juvenile/young animals also needs further investigation. Theoretically, the young animals should have higher lean muscle mass: body weight ratios and, therefore, higher creatinine excretion compared to mature animals, where the percentage of protein decreases and the percentage of fat increases in the mature animals.

Serum sodium had a strong positive correlation with serum chloride (Table 12). Chloride regulation in the blood is passively related to the sodium level and plays a role in the reabsorption of sodium from the ascending limb of Henle and distal tubule under the influence of aldosterone (Collins 1976). Serum sodium and chloride levels will, therefore, be closely correlated.

Urine SG and osmolarity had a very close positive correlation (Table 12). Urine SG is defined as the ratio of the density of urine to the density of distilled water, and a measure of total soluble solids (Muscat et al. 2011). The most abundant molecules in urine are urea, electrolytes, creatinine and other metabolic waste products (Muscat et al. 2011). Urine osmolarity is the concentration of osmotically active particles per unit of water in urine (osmolarity n.d.). Therefore, by definition, urine SG and urine osmolarity are very similar measures and are closely correlated.

There was a strong negative correlation between urine aldosterone and urine creatinine (Table 12). Gardner et al. (2006) and Wu et al. (2013) performed studies to determine if the urine aldosterone: creatinine ratio was a good alternative

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diagnostic tool compared to 24 hour urinary aldosterone levels in primary aldosteronism patients. It was found that 24 hour urinary aldosterone secretion had a highly significant correlation with urine aldosterone: creatinine ratio in dogs and humans (Gardner et al. 2006, Wu et al. 2013). Creatinine clearance provides an estimation of glomerular filtration rate (GFR) (DiBartola 2010). Therefore, when the GFR decreases, the amount of creatinine excreted in the urine will also decrease. GFR is reduced, for example, by hypovolaemia (DiBartola 2010). This will stimulate the RAAS, which will increase the reabsorption of sodium to correct the hypovolaemia (Buranakarl, Mathur & Brown 2004; Ibrahim, Rosenberg & Hostetter 1997). Aldosterone is responsible for sodium reabsorption (Louden 2012), and increased amounts of aldosterone will, therefore, appear in the urine. Thus, when urine creatinine decreases, urine aldosterone will increase. This is a negative correlation which is consistent with the findings in this study.

An unexpected correlation was the strong negative correlation between urine sodium and urine osmolarity (Table 12). Urinary urea, which was not measured in this study, is osmotically active in urine and is likely to play a critical role in urine osmolarity. Tauson et al. (2001) did a study which showed that the feeding of a high protein diet to mink resulted in an increase in urinary 24 hour excretion of urea. High protein diets have also been shown to result in significantly higher urine SG values in cats (Backlund et al. 2011). The greater excretion of urea in urine may thus be associated with low urinary sodium excretion. The reason for this, however, remains unclear and warrants further investigation.

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5.1. Limitations of Study

In this study, there were several limitations which need to be considered. The size of the study population was a major limitation. This data set contains only captive cheetahs from the AfriCat Foundation and free-ranging cheetahs from the Khomas and Omaheke districts in Namibia. Cheetahs from other locations may have somewhat different serum reference values. The small number of urine samples that were available, particularly of the free-ranging cheetah population, made statistical analysis between captive and free-ranging cheetahs impossible. Future studies may benefit from a larger study population, and from a wider and/or multiple geographical location(s).

It is possible that there could have been bias as the study population consisted mostly of male cheetahs, and the difference in age distribution of the captive and free-ranging cheetahs may have contributed to this bias. It is recommended, therefore, to have equal numbers of male and female cheetahs with equal numbers of young, mature and aged individuals.

Furthermore, reference intervals for only a selection of serum biochemistry analytes were established. The establishment of reference intervals for haematological and other various serum biochemistry analytes is recommended. The reference intervals for this cheetah population are only valid for the methodology and laboratory used in this study. There is variation between laboratories, different test methodologies, statistical analyses, sample handling and the evaluation and criteria of a 'healthy' cheetah (Lumsden 1998; Harr 2006) which means that the adoption of published reference intervals like these should be done with care. In addition, due to the high

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prevalence of renal disease in captive cheetahs, particularly glomerulosclerosis, there may be individuals in the study population suffering from renal disease which will influence the reference intervals calculated. However, these reference intervals can be applied to other populations after transference or validation procedures are performed according to CLSI and IFCC standards (Geffré et al. 2009).

It is recommended to immobilise the study population with the same immobilisation drugs and doses; and to standardise the collection, handling and centrifugation of the serum samples. It is also recommended that haemoglobin be measured quantitatively in the serum samples to accurately measure the degree of haemolysis and to standardise this scoring system.

This study would have benefited from a complete comparative nutritional analysis of the diet fed to the captive cheetahs in comparison to the diet consumed by the free-ranging cheetahs to determine any deficiencies and/or excesses of certain macro- and micro-nutrients. A feeding trial with different levels of sodium chloride and the effect this would have had on the serum and urine electrolyte levels and the renal physiological analytes in captive cheetahs would be ideal to investigate the possibility of a sodium deficiency in the diet of captive cheetahs.

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5.2. Conclusion

This is the first report of baseline reference intervals for serum biochemistry analytes of healthy captive and free-ranging cheetahs in Namibia. These reference intervals can be considered a useful tool for veterinary practitioners, researchers, zoologists and any other profession working in cheetah medicine and conservation. The data obtained for the urine analytes can also be used to aid in the assessment of health status of captive and free-ranging cheetahs. These results will aid in the accurate assessment of health and in the management of disease in cheetahs. Further research into the pathophysiology of diseases affecting cheetahs, using comprehensive health assessment data, is required to fully understand the threats facing this vulnerable species.

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Addendum A

Raw Data

ID	Name	Sex	Age	Weight (kg)	Haemolysis	Captive/Wild	Ssodium	Spotassium	Smagnesium	Schloride	BldUreaN	Screatinine	Sosmolarity	Ualdosterone	UrineSG	Uosmolarity	UProtToCreat
1	Quattro	Male	4	38.3	0	Captive								12.2	1.053	2210	0.01056
2	Spitfire	Male	4	48.5	1	Captive	157	4.93	0.93	123	16.5	192		6.29375	1.048	1919	0.02729
3	Charlie	Male	8	29.9	1	Captive	152.9	4.97	1.09	121.6	12	150		74.64166	1.06	2754	0.01242
4	Peanut	Male	7	45.7	1	Captive	153.4	4.34	0.96	121.1	14.2	222	321	3.445	1.06	2104	0.00236
5	Curly	Female	11	29.6	0	Captive	154.6	4.16	1.09	122.5	15.7	137		121.9883	1.06	2668	0.01467
6	Tumble	Male	6	42.1	1	Captive	150.3	4.51	0.93	119.5	11.6	204		4.8431	1.058	2536	0.00517
7	Ruff	Male	6	40.2	0	Captive	155.6	4.56	1.01	123.5	12	194	326	5.79074	1.054	2185	0.00416
8	Raisin	Male	7	38	2	Captive	153.2	4.66	1.08	121.7	14.4	180		3.3	1.053	2081	0.00438
9	Starsky	Male	5	36.4	0	Captive								5.38833	1.06	2448	0.00412
11	Morticia	Female	11	33.6	2	Captive	155.2	4.98	1.11	122.6	15.6	222	331	13.70689	1.058	2371	0.01618
12	Nip	Female	4	32.2	1	Captive	154.4	4.2	1.06	119.8	18.5	123			1.035		0.01665
13	Pepper	Female	7	40	0	Captive	154.5	4.89	1.08	121.8	14.7	201		8.48	1.055	2198	0.00371
14	Sugar	Female	7	38.4	1	Captive	158.1	5.01	1.04	125.2	14.5	273	330	6.36	1.055	1922	0.01154
15	Savannah	Female	4	36.2	0	Captive								9.90263	1.038	1652	0.00686
16	Salt	Male	7	45.7	0	Captive	154.3	4.23	1	121.1	14.9	186	318	10.17936	1.063	2778	0.00292
17	Ducati	Female	5	34.8	1	Captive	154.1	4.72	1.02	121.7	16.4	156		7.00357	1.056	2415	0.01838
18	Toby	Male	11	39	1	Captive								6.5619	1.042	1597	0.075
19	Buttons	Female	11	30.5	1	Captive	141.5	3.89	0.95	111.2	16.1	111	328	16.22448	1.049	2040	0.01619
23	Tuc	Male	4	41.5	1	Captive	154.2	5.07	1.02	117.3	16.9	197			1.03		0.05295
24	Tintin	Male	10	44.8	0	Captive	158.2	4.57	0.98	125.2	12	190			1.047		0.03143
25	Spice	Male	7	46.3	0	Captive	156	4.49	0.95	124.2	13.3	189			1.052		
26	Lucky	Female	13	32.6	0	Captive	154.4	3.89	1.01	124.9	20	174	327		1.068	2956	0.02577
28	Domino	Male	13	41.3	0	Captive	153.5	4.89	1.07	122.9	16.1	222	322				
30	Dash	Female	6	30.7	1	Captive	153.8	4.71	0.99	126	10.5	165	319		1.075		

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ID	Name	Sex	Age	Weight (kg)	Haemolysis	Captive/Wild	Ssodium	Spotassium	Smagnesium	Schloride	BldUreaN	Screatinine	Sosmolarity	Ualdosterone	UrineSG	Uosmolarity	UProtToCreat
31	Abbey	Female	10	33.5	1	Captive	155.1	4.27	1.09	122.1	13.2	170			1.048		
32	R	Female	14	32	0	Captive	152.7	4.34	1.01	123.2	24.6	213					
34	Sniper	Male	4	34.5	0	Captive									1.048	2082	0.00943
36	Bubbles	Male	11	38.2	1	Captive	152.8	4.42	1.09	120	17	152					
37	Vax	Female	9	35.9	1	Captive	155	4.37	1.01	121.5	10.8	180			1.043	1549	0.03386
39	Scamp	Male	4	40.7	0	Captive	155.6	4.67	1.06	116.7	18.5	136			1.057		0.01745
40	Dyson	Male	9	41.1	0	Captive	157.3	4.18	0.91	125.9	14.2	217	331		1.048	1878	0.01099
41	Hoover	Female	9	34.4	0	Captive	153.1	4.6	1	121.9	16.5	151	324		1.063	2773	0.02561
42	Pugsley	Male	12	38.1	0	Captive	155.7	4.41	1.1	122.4	18.5	281					
43	Gomez	Male	12	36	0	Captive	152.9	4.11	1.16	118.9	26.2	164	334		1.058	2607	0.01736
44	Wednesday	Female	12	32	1	Captive	153.6	4.2	1.21	122.4	21.9	156					
91	Aprillia	Female	5	35.7		Captive									1.053		0.02448
20	Coco	Female	8	32.5	1	Wild	158.9	4.77	1.07	127.1	11.2	181	336		1.08		0.00515
21	Spud	Male	8	35.6	1	Wild	156.5	4.32	0.92	123.6	15.6	108					
22	Bones	Male	8	39	1	Wild	157.7	4.19	1.1	123.4	9.3	141	326		1.065	2863	0.01073
45	P119	Male	3-4 months		1	Wild									1.026	1244	0.0705
46	A076	Female	4-4.5 years		1	Wild	159.8	5.19	1.18	121.3	11.3	183					
47	A133A	Male	3-4years		2	Wild	155.1	4.54	1.07	118.8	17.5	85					
48	P111	Male	2 years		2	Wild	160.8	4.71	1.04	124.6	13.9	273					
49	A119	Male	3 years		2	Wild	160.3	4.34	1.17	120.1	8.3	138					
50	P075	Male	5-6 years		1	Wild	157.8	4.41	1.07	121.6	9	201					
52	P116	Female	8-9months		3	Wild	155.6	4.41	0.97	118.8	20	121	330				
53	W053A	Male	5-6 years		2	Wild	154.2	3.95	1.1	114	18	100					
54	A112A	Male	5.5-6 years		2	Wild	157.8	4.58	1	122.9	11.7	143					
55	P121	Male	2-3 years		2	Wild	155.4	4.16	0.94	121.7	8.3	190					

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ID	Name	Sex	Age	Weight (kg)	Haemolysis	Captive/Wild	Ssodium	Spotassium	Smagnesium	Schloride	BldUreaN	Screatinine	Sosmolarity	Ualdosterone	UrineSG	Uosmolarity	UProtToCreat
56	P120	Male	Adult		2	Wild	175.6	3.9	1.2	137.8	12.4	150					
57	A120	Male	10 months		2	Wild	136.9	4.38	0.86	105	9.6	209					
58	P118	Male	3-4 months		2	Wild									1.026		0.12603
61	A131	Male	18-20 months		2	Wild	123.5	4	0.82	97.2	13.3	183					
62	W053B	Male	5.5-6 years		2	Wild	166.8	4.1	1.13	129.9	16.9	142	350		1.056		
63	P114	Female	2-2.5 years		4	Wild	153.9	5.16	0.97	115.8	18.6	169	363				
64	A122	Male	10 months		3	Wild	133.6	3.8	0.84	102.9	9	197					
65	W053C	Male	5.5-6 years		3	Wild	147	4.01	1.07	114.3	16	144					
66	P123	Male	3-4 years		3	Wild	157.3	5	0.92	121.9	10.2	232					
69	P112	Male	2 years		3	Wild	158.9	4.86	0.94	122.7	12	234	326				
71	A077	Male	5.5-6 years		1	Wild	144.5	4.02	1.04	110.3	10.6	178					
72	P073	Male	5.5-6 years		2	Wild	154.4	4.11	0.9	111.3	9.6	209					
75	P115	Male	8-9 months		3	Wild	150.5	5.11	1.02	110	19.3	124					
76	P110	Male	2 years		1	Wild	165.1	4.72	1.03	129.3	8.3	141					
77	P122	Male	3-4 years		2	Wild	157.6	4.45	0.98	121.8	8.4	152					
78	A137	Male	3-4 years		1	Wild	153.5	4.39	0.9	118.5	8.9	211					
79	A138	Female	4-5 years		2	Wild	162	4.54	1.11	126.7	9.5	164					
80	A135	Female	3.5-4 years		2	Wild	159.1	4.69	1.02	125.3	10.7	158					
81	A132	Male	18-20 months		1	Wild	152.7	4.97	1.18	123.5	11.4	163					
82	P113	Female	8-9 months		1	Wild	129.7	4.58	0.82	96.1	16	90	281				
83	A121	Male	10 months		3	Wild	160.1	5.27	1.04	121.3	11.7	303					
86	A136	Female	15-17 months		2	Wild	153.2	5.11	0.96	117.4	13.2	199					
87	P126	Female	3-4 years		3	Wild	158.2	4.49	1.09	125.2	12.8	162					
88	K004	Male	2-3 years		1	Wild	161.4	5.01	1.04	124.3	10.4	146	330				
89	A112B	Male	5.5-6 years		1	Wild	163.7	4.51	1.1	129.3	8.1	147					

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Addendum B

Spearman's Correlation Co-efficients

	Usodium	Upotassium	Umagnesium	Uchloride	UricAcid	Uprotein	Ucreatinine	Ualdosterone	UrineSG	Uosmolarity	Ssodium	Spotassium	Smagnesium	Schloride	BldUreaN	Screatinine	Sosmolarity	UProtToCreat
Usodium	1	-0.04945	0.16781	0.23077	0.3416	0.29986	-0.03297	0.08571	-0.55725	-0.97619	0.52696	0.47619	-0.33737	0.61905	-0.27545	0.02381	0.4	0.23626
Usodium		0.8725	0.5837	0.4481	0.2533	0.3195	0.9149	0.8717	0.0479	<.0001	0.1796	0.2329	0.4138	0.1017	0.5091	0.9554	0.6	0.4371
	13	13	13	13	13	13	13	6	13	8	8	8	8	8	8	8	4	13
Upotassium	-0.04945	1	-0.26229	-0.30567	0.23046	0.34894	0.25	-0.0098	-0.00117	-0.13308	0.49119	0.15134	0.00871	0.2506	-0.41411	0.16616	0.49825	0.1611
Upotassium	0.8725		0.1403	0.0836	0.1969	0.0466	0.1606	0.9702	0.9948	0.526	0.0148	0.4802	0.9678	0.2376	0.0442	0.4378	0.0992	0.3705
	13	33	33	33	33	33	33	17	33	25	24	24	24	24	24	24	12	33
Umagnesium	0.16781	-0.26229	1	0.27025	-0.36129	-0.25665	-0.20812	0.06009	0.28452	0.19315	-0.32398	-0.03023	0.25365	-0.06049	-0.04834	-0.01262	0.27944	-0.14393
Umagnesium	0.5837	0.1403		0.1282	0.0388	0.1494	0.2451	0.8188	0.1085	0.3549	0.1225	0.8885	0.2317	0.7789	0.8225	0.9533	0.3791	0.4242
	13	33	33	33	33	33	33	17	33	25	24	24	24	24	24	24	12	33
Uchloride	0.23077	-0.30567	0.27025	1	-0.38782	-0.22406	-0.34712	0.11281	-0.02111	0.05578	-0.1662	0.03175	-0.27582	-0.15575	0.27172	-0.0696	-0.27719	-0.02022
Uchloride	0.4481	0.0836	0.1282		0.0257	0.21	0.0478	0.6664	0.9072	0.7911	0.4377	0.8829	0.192	0.4674	0.199	0.7466	0.3831	0.9111
	13	33	33	33	33	33	33	17	33	25	24	24	24	24	24	24	12	33
UricAcid	0.3416	0.23046	-0.36129	-0.38782	1	0.56639	0.15163	0.03085	-0.12037	-0.20783	0.28478	0.33806	0.08408	0.46991	-0.31318	0.13473	-0.07788	0.35782
UricAcid	0.2533	0.1969	0.0388	0.0257		0.0006	0.3996	0.9064	0.5046	0.3188	0.1774	0.1062	0.6961	0.0205	0.1362	0.5302	0.8099	0.0409
	13	33	33	33	33	33	33	17	33	25	24	24	24	24	24	24	12	33
Uprotein	0.29986	0.34894	-0.25665	-0.22406	0.56639	1	-0.07524	0.08482	-0.37682	-0.27141	0.39369	0.37938	-0.02855	0.31882	0.04008	0.09726	0.40702	0.85571
Uprotein	0.3195	0.0466	0.1494	0.21	0.0006		0.6773	0.7462	0.0306	0.1894	0.057	0.0675	0.8946	0.1289	0.8525	0.6512	0.1891	<.0001
	13	33	33	33	33	33	33	17	33	25	24	24	24	24	24	24	12	33
Ucreatinine	-0.03297	0.25	-0.20812	-0.34712	0.15163	-0.07524	1	-0.62255	0.21393	-0.03077	0.39808	0.27125	-0.25555	0.44572	-0.75452	0.63646	-0.17181	-0.5137
Ucreatinine	0.9149	0.1606	0.2451	0.0478	0.3996	0.6773		0.0076	0.2036	0.8839	0.0359	0.1627	0.1893	0.0174	<.0001	0.0003	0.557	0.0022
	13	33	33	33	33	33	37	17	37	25	28	28	28	28	28	28	14	33
Ualdosterone	0.08571	-0.0098	0.06009	0.11281	0.03085	0.08482	-0.62255	1	0.09759	0.30637	-0.01099	-0.06044	0.46897	-0.04132	0.40165	-0.50619	0.42857	0.39461
Ualdosterone	0.8717	0.9702	0.8188	0.6664	0.9064	0.7462	0.0076		0.7094	0.2317	0.9716	0.8445	0.106	0.8934	0.1737	0.0776	0.3965	0.117
	6	17	17	17	17	17	17	17	17	17	13	13	13	13	13	13	6	17
UrineSG	-0.55725	-0.00117	0.28452	-0.02111	-0.12037	-0.37682	0.21393	0.09759	1	0.93185	-0.15857	-0.06473	0.20745	0.20398	-0.12766	-0.15279	-0.30132	-0.45846
UrineSG	0.0479	0.9948	0.1085	0.9072	0.5046	0.0306	0.2036	0.7094		<.0001	0.4203	0.7435	0.2895	0.2978	0.5174	0.4376	0.2951	0.0073
	13	33	33	33	33	33	37	17	37	25	28	28	28	28	28	28	14	33
Uosmolarity	-0.97619	-0.13308	0.19315	0.05578	-0.20783	-0.27141	-0.03077	0.30637	0.93185	1	-0.27205	-0.24748	0.37852	-0.05707	0.20817	-0.45479	-0.37443	-0.22
Uosmolarity	<.0001	0.526	0.3549	0.7911	0.3188	0.1894	0.8839	0.2317	<.0001		0.2598	0.307	0.11	0.8165	0.3924	0.0504	0.2566	0.2906
	8	25	25	25	25	25	25	17	25	25	19	19	19	19	19	19	11	25
Ssodium	0.52696	0.49119	-0.32398	-0.1662	0.28478	0.39369	0.39808	-0.01099	-0.15857	-0.27205	1	0.21075	0.30327	0.69314	-0.33631	0.01057	0.45602	0.02741
Ssodium	0.1796	0.0148	0.1225	0.4377	0.1774	0.057	0.0359	0.9716	0.4203	0.2598		0.0894	0.0133	<.0001	0.0058	0.9329	0.0433	0.8988
	8	24	24	24	24	24	28	13	28	19	66	66	66	66	66	66	20	24
Spotassium	0.47619	0.15134	-0.03023	0.03175	0.33806	0.37938	0.27125	-0.06044	-0.06473	-0.24748	0.21075	1	0.03546	0.15319	-0.05191	0.23866	0.05814	0.07654
Spotassium	0.2329	0.4802	0.8885	0.8829	0.1062	0.0675	0.1627	0.8445	0.7435	0.307	0.0894		0.7774	0.2194	0.6789	0.0536	0.8077	0.7222
	8	24	24	24	24	24	28	13	28	19	66	66	66	66	66	66	20	24
Smagnesium	-0.33737	0.00871	0.25365	-0.27582	0.08408	-0.02855	-0.25555	0.46897	0.20745	0.37852	0.30327	0.03546	1	0.30226	0.13249	-0.26232	0.39305	-0.09499
Smagnesium	0.4138	0.9678	0.2317	0.192	0.6961	0.8946	0.1893	0.106	0.2895	0.11	0.0133	0.7774		0.0136	0.2889	0.0334	0.0865	0.6588
	8	24	24	24	24	24	28	13	28	19	66	66	66	66	66	66	20	24
Schloride	0.61905	0.2506	-0.06049	-0.15575	0.46991	0.31882	0.44572	-0.04132	0.20398	-0.05707	0.69314	0.15319	0.30226	1	-0.19165	0.08358	0.23547	-0.11138
Schloride	0.1017	0.2376	0.7789	0.4674	0.0205	0.1289	0.0174	0.8934	0.2978	0.8165	<.0001	0.2194	0.0136		0.1232	0.5046	0.3176	0.6044
	8	24	24	24	24	24	28	13	28	19	66	66	66	66	66	66	20	24
BldUreaN	-0.27545	-0.41411	-0.04834	0.27172	-0.31318	0.04008	-0.75452	0.40165	-0.12766	0.20817	-0.33631	-0.05191	0.13249	-0.19165	1	-0.19015	0.27087	0.45548
BldUreaN	0.5091	0.0442	0.8225	0.199	0.1362	0.8525	<.0001	0.1737	0.5174	0.3924	0.0058	0.6789	0.2889	0.1232		0.1262	0.248	0.0253
	8	24	24	24	24	24	28	13	28	19	66	66	66	66	66	66	20	24
Screatinine	0.02381	0.16616	-0.01262	-0.0696	0.13473	0.09726	0.63646	-0.50619	-0.15279	-0.45479	0.01057	0.23866	-0.26232	0.08358	-0.19015	1	-0.01209	-0.30013
Screatinine	0.9554	0.4378	0.9533	0.7466	0.5302	0.6512	0.0003	0.0776	0.4376	0.0504	0.9329	0.0536	0.0334	0.5046	0.1262		0.9597	0.1542
	8	24	24	24	24	24	28	13	28	19	66	66	66	66	66	66	20	24
Sosmolarity	0.4	0.49825	0.27944	-0.27719	-0.07788	0.40702	-0.17181	0.42857	-0.30132	-0.37443	0.45602	0.05814	0.39305	0.23547	0.27087	-0.01209	1	0.33684
Sosmolarity	0.6	0.0992	0.3791	0.3831	0.8099	0.1891	0.557	0.3965	0.2951	0.2566	0.0433	0.8077	0.0865	0.3176	0.248	0.9597		0.2843
	4	12	12	12	12	12	14	6	14	11	20	20	20	20	20	20	20	12
UProtToCreat	0.23626	0.1611	-0.14393	-0.02022	0.35782	0.85571	-0.5137	0.39461	-0.45846	-0.22	0.02741	0.07654	-0.09499	-0.11138	0.45548	-0.30013	0.33684	1
UProtToCreat	0.4371	0.3705	0.4242	0.9111	0.0409	<.0001	0.0022	0.117	0.0073	0.2906	0.8988	0.7222	0.6588	0.6044	0.0253	0.1542	0.2843	
	13	33	33	33	33	33	33	17	33	25	24	24	24	24	24	24	12	33

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Addendum C

Journal Publication Emanating from this Research

Title: Reference intervals for selected serum biochemistry analytes in cheetahs (*Acinonyx jubatus*)

Authors: Hudson-Lamb G.C., Schoeman J.P., Hooijberg E.H., Heinrich S.K. and Tordiffe A.S.W.

Journal: Journal of the South African Veterinary Association (JSAVA)

Status: Accepted for publication (16 October 2015)

Abstract

Published haematologic and serum biochemistry reference intervals are very scarce for captive cheetahs, and more so for free-ranging cheetahs. The current study was performed to establish reference intervals for selected serum biochemistry analytes in cheetahs. Baseline serum biochemistry analytes were analysed from 66 healthy Namibian cheetahs. Samples were collected from 30 captive cheetahs at the AfriCat Foundation, and 36 free-ranging cheetahs from central Namibia. The effects of captivity-status, age, sex and haemolysis score on the tested serum analytes was investigated. The biochemical analytes that were measured were sodium, potassium, magnesium, chloride, urea and creatinine. The 90% confidence interval of the reference limits were obtained using the non-parametric bootstrap method. Reference intervals were preferentially determined by the non-parametric method and were as follows: sodium (128 – 166 mmol/l), potassium (3.9 – 5.2 mmol/l),

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magnesium (0.8 – 1.2 mmol/l), chloride (97 – 130 mmol/l), urea (8.2 – 25.1 mmol/l) and creatinine (88 – 288 µmol/l). Reference intervals from the current study were compared with International Species Information System values for cheetahs and were found to be narrower. Moreover, age, sex and haemolysis score had no significant effect on the serum analytes in this study. Separate reference intervals for captive and free-ranging cheetahs were also determined. Captive cheetahs had higher urea values, most likely due to dietary factors. This study is the first to establish reference intervals for serum biochemical analytes in cheetahs according to international guidelines. These results can be used for future health and disease assessments in both captive and free-ranging cheetahs.

Introduction

The cheetah (*Acinonyx jubatus*) is the last remaining member of the genus *Acinonyx* and is listed as vulnerable on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN 2014). Perspectives gleaned from veterinary science can be incorporated as part of a multidisciplinary approach to conservation, where it can assist in the successful planning, implementation and evaluation of conservation projects (Karesh & Cook 1995). The measurement of blood analytes forms a major role in the assessment of the health of an individual or population. Species-specific haematological and serum biochemical reference intervals are arguably one of the most powerful tools in veterinary medicine to aid in the clinical decision-making process of making diagnoses and the management of disease (Friedrichs et al. 2012).

There are only a handful of publications that provide information on haematological

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and serum biochemistry analytes in cheetahs (Bechert et al. 2002; Caro et al. 1987; Depauw et al. 2012; Holder et al. 2004). These studies provide values mainly for captive cheetahs. One study also provides additional values for free-ranging cheetahs in East Africa (Caro et al. 1987).

In addition, unpublished data are available from the International Species Information System (ISIS). These data form part of an electronic database of animals held in zoological institutions to which member institutions contribute health and genetic data (ISIS 2002). These values are presented as means, standard deviations, and minimum and maximum values.

The reference values from both published and unpublished sources do not comply with the guidelines for the generation of reference intervals in veterinary species, as stipulated by the American Society for Veterinary Clinical Pathology (ASVCP), which in turn is based on guidelines from the International Federation of Clinical Chemistry (IFCC) and the Clinical and Laboratory Standards Institute (CLSI) (Friedrichs et al. 2012). In view of this, the aim of this study was to establish reference intervals according to these guidelines for selected serum biochemistry analytes in healthy cheetahs. The effect of age, sex, haemolysis and captivity-status on the serum analytes and the establishment of separate reference intervals for captive and free-ranging cheetahs was investigated.

Research method and design

The study population consisted of 66 cheetahs (*Acinonyx jubatus*), of which 30 were captive cheetahs and 36 were free-ranging cheetahs. During June – July 2013, the

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captive cheetahs were immobilised and sampled at the AfriCat Foundation, Okonjima, Namibia, during their annual veterinary health examinations. The free-ranging cheetahs were trapped, immobilised and sampled on communal and commercial farmland in the Khomas and Omaheke districts, central Namibia. Animals that were used in the study were clinically healthy as far as could be determined according to history and clinical examination.

The captive cheetahs were immobilised by remote intramuscular injection, and the free-ranging cheetahs were captured in box traps placed at cheetah marking trees and then immobilised by remote intramuscular injection. Immobilisation was achieved with a combination of 0.03 mg/kg medotomidine (Medotomidine - 10 mg/ml, Kyron Laboratories, Johannesburg, South Africa, 2094) and 1.2 mg/kg zolazapam/tiletimine (Zoletil®, Virbac Animal Health, Centurion, South Africa, 0157) for the captive animals, and 0.08 mg/kg medetomidine and 4.5 mg/kg ketamine (Ketamine 1G, Kyron Laboratories, Johannesburg, South Africa, 2094) for the free-ranging animals injected intramuscularly. Free-ranging cheetahs were reversed with 0.25 mg/kg atipamezole and released again.

During sample collection, the sex and age of the cheetahs were recorded. The age of the captive cheetahs was transcribed from accurate records that were available at the AfriCat Foundation. The age of the free-ranging cheetahs was estimated by using the key for body size established by Caro (1994) for East African cheetahs. This was performed by evaluating shoulder height, appearance of the mane, dental wear and physical lesions such as elbow calluses and scars.

Within 15 minutes of immobilisation, serum samples were obtained from the captive cheetahs by collecting 10 ml of whole blood from the jugular vein with a 20 ml

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syringe and 18 G needle. The blood was then transferred into serum BD Vacutainer® tubes (Becton, Dickinson and Company, Woodmead, Johannesburg, South Africa, 2191) and allowed to clot for 40 minutes on ice. After centrifuging the samples at 1700 g for five minutes, the serum was pipetted off into 1.8 ml Cryovials® (Thermo Scientific, Germiston, South Africa, 1401) and frozen at -20 °C. In the free-ranging cheetahs, blood was collected directly into serum BD Vacutainer® tubes. The samples were placed on ice for between 4 and 24 hours until they could be centrifuged at 400 g for 15 minutes. The serum was then separated from the cells and frozen at -20 °C. Haemolysis scoring of the serum samples was done by means of visual assessment and rated as 0 (no haemolysis), 1+ (mild haemolysis) and > 1+ (moderate – severe haemolysis) after centrifugation was performed. Samples remained frozen until time of transportation where they were transported within 24 hours in a liquid nitrogen canister to the National Zoological Gardens of South Africa (NZG), Pretoria, South Africa. The samples were stored at -80 °C at the NZG, and were then transported on dry ice within 30 minutes to the Clinical Pathology Laboratory, Onderstepoort Veterinary Academic Hospital, Faculty of Veterinary Science, Onderstepoort Campus, University of Pretoria, Pretoria, where all the biochemical analyses were performed.

The Cobas Integra 400 Plus Analyser® (Roche, Illovo, Johannesburg, South Africa, 1609) was used to measure serum sodium, potassium, magnesium, chloride, urea and creatinine. The quantitative determination of serum sodium, potassium and chloride was achieved using ion-selective electrodes (ISE indirect method). Serum magnesium concentrations were determined using a colorimetric method with Chlorophosphonazo III. Serum urea determinations were performed by a kinetic test with urease and glutamate dehydrogenase. Serum creatinine concentrations were

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measured using a buffered kinetic Jaffé reaction without deproteinisation. The within-laboratory coefficient of variation for each variable was as follows: sodium, 1.1%; potassium, 1.1%; magnesium, 2.5%; chloride, 1.8%; urea, 3.1%; and creatinine, 3.4%. Both daily internal and monthly external quality control was performed on this analyser and results fell within the laboratory's pre-set performance goals.

The Mann-Whitney U (MWU) test was used to determine significant differences between the captive and free-ranging cheetah populations with regard to the age distribution and the serum analytes. Chi-Square tests were performed on sex and haemolysis scores to determine significant differences between the captive and free-ranging cheetahs. To determine if there was an association between the age, sex or haemolysis score with serum biochemical test results, Spearman's rank correlation co-efficient was calculated for age, the MWU test for sex and the Kruskal-Wallis test for haemolysis score. p-values < 0.05 and Spearman's correlation co-efficient of $r_s > 0.6$ or < -0.6 were considered significant (Weir n.d.).

The establishment of the reference intervals was guided by recommendations published by the ASVCP (ASVCP 2015), based in turn on guidelines from the IFCC and the CLSI (Friedrichs et al. 2012). Common descriptive statistics for all serum analytes included sample size, mean, median, standard deviation, minimum and maximum values. The variables were tested for Gaussian distribution according to the Anderson-Darling test with a significance level of 5%, as well as by visual inspection using histograms and Q-Q plots. The Dixon-Reed and Tukey's tests were used to test for and identify outliers, and emphasis was on retaining rather than deleting outliers. The 90% confidence interval of the reference limits was obtained using the non-parametric bootstrap method. Reference intervals were preferentially determined by the non-parametric method, which is independent of the distribution of

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the data. Where non-parametric methods could not be used, reference intervals were based on a robust method, preferably after Box-Cox transformation of the data to a distribution that was Gaussian.

The statistical software that was used to analyse the data was SAS®, Version 9.3; Statistica®, Version 12; and Reference Value Advisor®, Version 2.1 (for Microsoft Office®, 2010) (Geffré et al. 2011).

Results

Six serum biochemical analytes were measured in 66 healthy captive (n = 30) and free-ranging (n = 36) cheetahs, consisting of 43 males and 23 females. There were significantly more male cheetahs in the free-ranging population (75%) than in the captive one (53%) (Chi-Square test, $p = 0.0499$) (Table 1). The captive cheetahs were significantly older than the free-ranging cheetahs (Mann-Whitney U test, $p < 0.01$) (Table 1), with the median age of captive cheetahs (seven years) being almost twice that of the free-ranging cheetahs sampled (four years). There was significantly more haemolysis in the free-ranging cheetah samples compared to the captive cheetah samples (Chi-Square test, $p < 0.01$) (Table 2).

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Table 1: Summary of sex and age distributions of captive and free-ranging Namibian cheetahs. Age data are reported for inter-quartile range, mean ± standard deviation (SD), and median for all animals.

	Captive	Free-ranging
Sex (*n*)		
Male	16	27
Female	14	9
Age (years)		
Inter-quartile Range	5 – 11	2 – 6
Mean ± SD	8.5 ± 3.0	4.0 ± 2.2
Median	7	4

Table 2: Comparison of haemolysis scores of serum samples between captive and free-ranging Namibian cheetahs.

Haemolysis score	Captive	Free-ranging	Total
0†	14	0	14
1+‡	14	12	26
>1+§	2	24	26
Total	30	36	66
† No haemolysis			
‡ Mild haemolysis			
§ Moderate–severe haemolysis			

Visual inspection of the data and the Anderson-Darling normality test showed that four of the six variables were normally distributed (potassium, magnesium, urea and creatinine). In contrast, the distribution of data was non-Gaussian for sodium and chloride, and did not show Gaussian distribution even after Box-Cox transformation. Both data sets were skewed to the left. A single outlier was excluded in the

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calculation of the reference intervals for sodium and chloride, based on visual inspection of the data rather than using the Tukey or Dixon-Reed criteria, which only listed this value as suspect on the non-transformed data. The outlier for both sodium and chloride belonged to the same individual which had a very high sodium (175.6 mmol/l) and chloride (137.8 mmol/l) concentration. No outliers were excluded from other variables.

Reference intervals of the study population were calculated using the non-parametric method for serum sodium, potassium, magnesium, chloride, urea and creatinine (Table 3) and are compared with the ISIS values for all cheetahs in the ISIS database (mean, standard deviation, and minimum and maximum value) (ISIS 2002). Partitioning criteria were applied to the captive and free-ranging cheetahs where separate reference intervals were determined using the robust method after Box-Cox transformation for all the measured serum analytes, except for urea of the captive population where a robust method was used on untransformed data (Table 4).

The median sodium concentration was significantly lower and the median urea concentration was significantly higher in captive cheetahs versus free-ranging cheetahs (MWU test, $p < 0.05$) (Table 4). The median sodium concentration differed by 3 mmol/l between captive (154 ± 3 mmol/l) and free-ranging cheetahs (157 ± 10 mmol/l), and the median urea concentration of captive cheetahs (15.7 ± 3.7 mmol/l) was higher than that of free-ranging cheetahs (11.4 ± 3.5 mmol/l) (Table 4).

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Table 3: Reference intervals for six serum analytes in Namibian cheetahs in the present study and ISIS values for all cheetahs.

Serum Analyte		Study Population							Cheetah ISIS Values (2002)				
	Unit	*n*	Mean	SD	Median	Min	Max	RI	*n*	Mean	SD	Min	Max
Sodium	mmol/l	65	154	7	155	124	167	128–166	1066	157	4	128	175
Potassium	mmol/l	66	4.5	0.4	4.5	3.8	5.3	3.9–5.2	1068	4.4	0.5	3.1	7.0
Magnesium	mmol/l	66	1.0	0.1	1.0	0.8	1.2	0.8–1.2	26	1.0	0.1	0.8	1.5
Chloride	mmol/l	65	120	7	122	96	130	97–130	1015	122	4	108	136
Urea	mmol/l	66	13.9	4	13.3	8.1	26.2	8.2–25.1	1105	12.9	3.2	5.3	29.6
Creatinine	µmol/l	66	175	44	172	85	303	88–288	839	212	80	53	716

Standard International (SI) units used

Min, Minimum; Max, Maximum; SD, Standard Deviation; RI, Reference Interval

Table 4: Means, standard deviations, medians, minimum and maximum values, and reference intervals for serum analytes in captive and free-ranging Namibian cheetahs, using the Mann-Whitney U Test.

Serum Analyte		Captive†						Free-ranging‡						
	Unit	Mean	SD	Median	Min	Max	RI	Mean	SD	Median	Min	Max	RI	*p* - value
Sodium	mmol/l	154	3	154	142	153	148–160	155	157	10	124	176	137–178§	0.02
Potassium	mmol/l	4.5	0.3	4.5	3.9	5.1	3.8–5.2	4.5	4.5	0.4	3.8	5.3	3.7–5.4	0.93
Magnesium	mmol/l	1.0	0.1	1.0	0.9	1.2	0.9–1.2	1.0	1.0	0.1	0.8	1.2	0.8–1.2	0.67
Chloride	mmol/l	122	3	122	111	126	116–129	119	122	9	96	138	103–140§	0.50
Urea	mmol/l	15.9	3.7	15.7	10.5	26.2	7.4–22.9	12.3	11.4	3.5	8.1	20.0	7.2–21.4	< 0.01
Creatinine	µmol/l	184	39	183	111	273	114–276	169	163	47	85	303	89–283	0.11

Standard International (SI) units used

SD, Standard Deviation; Min, Minimum; Max, Maximum; RI, Reference Interval

†, *n* = 30; ‡, *n* = 36; §, n = 35 (due to outlier that was excluded)

Ethical considerations

Research permits to conduct this study were obtained by the University of Pretoria's Research Committee (V033/13) and Animal Ethics Committee (V033/13), National Zoological Gardens Research Ethics and Scientific Committee (NZG/P13/26) and

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the Namibian Ministry of Environment and Tourism (1846/2013, 1689/2012 and 1813/2013).

Discussion

Most of the cheetahs in the study population were male (66.7%) (Table 1) due to the high proportion of males in the free-ranging population (76.9%). This is possibly due to the fact that box traps used to capture the free-ranging cheetahs were placed at cheetah marking trees which are more often frequented by males than females. Similarly, in a study by Marker et al. (2003), the demographics of free-ranging Namibian cheetahs was found to be biased towards the capturing of adult males, with 2.9 males captured for every adult female. This skewed sex ratio was attributed to a sampling bias, rather than a true indication of population structure in the wild.

The age demographics of the study population also differed, where the captive population was significantly older than the free-ranging population (Table 1). The AfriCat Foundation does not breed any captive carnivores, and almost all of the cheetahs at the facility were orphaned cubs which have subsequently been in captivity for a number of years already, with the resultant absence of young captive cheetahs. The highest peak of mortality in free-ranging cheetahs is between five and six years of age, with the maximum age recorded for a free-ranging Namibian cheetah being 12 years (Marker et al. 2003). This is consistent with the findings in this study where there were a small number (n = 4) of free-ranging cheetahs over six years of age.

The serum samples from free-ranging cheetahs were significantly more haemolysed

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than those from captive cheetahs (Table 2). The blood collection technique, as well as the time interval between sample collection and processing, differed for the samples collected in the captive and free-ranging cheetahs. Operator-related factors such as operator experience or sample handling times (Grant 2003; Ong, Chan & Lim 2009) could explain the difference in haemolysis observed.

Despite significant differences in age, sex and haemolysis scores between captive and free-ranging cheetahs, none of these variables had any significant effect on the serum biochemistry analytes for the study population as well as the captive and free-ranging sub-groups. Separate reference intervals based on these variables were, therefore, not calculated.

Reference intervals of the study population were compared with the ISIS values for all cheetahs (ISIS 2002) (Table 3). The mean ISIS values for the measured serum analytes all fell within the reference intervals that were calculated in this study and were very similar to the mean values of the study population, except for creatinine (212 µmol/l) which was 1.2 times higher than the study population (175 µmol/l). The minimum ISIS values fell outside the respective reference interval, except for sodium and chloride. The maximum ISIS values were all above the reference intervals for the serum analytes in this study. Interpretation of these findings is complicated by the fact that the ISIS values are unpublished data consisting of captive animals of unknown health status (ISIS 2002). Multiple samples may also be from the same individual and analytical methods are not described. Furthermore, normality of these data is not reported, thus it is unclear if these means and standard deviations can be used to establish reference intervals.

Separate reference intervals for the captive and free-ranging sub-groups were

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calculated according to ASVCP guidelines (ASVCP 2015) (Table 4). As there were less than 40 samples for each sub-group, the uncertainty and imprecision of the reference limits may be very high (Geffré et al. 2009). Nevertheless, there were large differences in the reference intervals for serum sodium, chloride and creatinine; where these analytes had reference intervals that were wider in the free-ranging cheetahs.

The free-ranging cheetahs may have a slightly higher median sodium value than captive cheetahs as a result of dehydration, since the free-ranging cheetahs were caught in box traps with no free access to water and a variable amount of time may have elapsed before samples were collected.

Pre-renal causes for elevated urea are increased protein catabolism (e.g. small bowel haemorrhage and necrosis, starvation, and prolonged exercise), dehydration and high protein diets (Backlund et al. 2011; DiBartola 2010). The captive cheetahs at the AfriCat Foundation are fed a high protein diet of 1.0 – 1.5 kg donkey meat on a daily basis, compared to their free-ranging counterparts that consume the muscle meat and viscera of small antelope, rodents and birds, and seldom eat daily (Bechert et al. 2002). The high frequency of feeding large portions of high protein meat may create an overall protein excess and elevated urea levels in comparison to free-ranging cheetahs. In a study by Caro et al. (1987) it was also found that captive cheetahs had higher urea levels than free-ranging cheetahs. This finding was attributed to a higher total food intake, where it was found that free-ranging cheetahs were consuming about 75% of the captive diet. The cheetahs in captivity have free access to water at all times, ruling out dehydration as a possible pre-renal cause of elevated urea. Other causes of increased protein catabolism are unlikely in the captive cheetah population as they were all fed daily and do not exercise for

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prolonged periods. The high protein diet could, however, mask elevated urea levels due to renal disease (renal azotaemia) and/or gastritis (small bowel haemorrhage) (DiBartola 2010). Lymphoplasmacytic gastritis and renal disease are two of the most prevalent diseases of captive cheetahs, and are rare in free-ranging cheetahs (Munson et al. 2005). Renal disease is a leading cause of morbidity and mortality in captive cheetahs, and renal lesions were present in 90% of a captive cheetah population in one study (Munson 1993). Glomerulosclerosis is one of the two main lesions seen in captive cheetahs with renal disease, the other being renal amyloidosis (Bolton & Munson 1999). Over 80% of captive cheetahs in the United States and South Africa suffer from glomerulosclerosis (Munson 1993). Bolton and Munson (1999) stated that the daily feeding of high protein diets to captive cheetahs may influence the development of glomerulosclerosis. Clinical pathology indices of renal dysfunction, such as serum creatinine and urea levels, are therefore highly relevant and essential in the detection of renal disease in cheetahs, particularly captive ones.

Limitations of the study

In this study, there were several limitations which need to be considered. The size of the study population was a major limitation. This data set contains only captive cheetahs from the AfriCat Foundation and free-ranging cheetahs from the Khomas and Omaheke districts in Namibia and the reference intervals generated apply to these populations and the analytical methods used. Cheetahs from other locations may have somewhat different serum reference values. Reference intervals may also differ based on the analytical methods used. Furthermore, due to the high

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prevalence of renal disease in captive cheetahs, particularly glomerulosclerosis, there may be individuals in the study population suffering from renal disease which will influence the reference intervals calculated. In order to use these reference intervals for other populations, transference or validation procedures should be performed according to CLSI and IFCC standards (Geffré et al. 2009).

Future studies may benefit from a larger study population, and from a wider and/or multiple geographical location(s). Furthermore, reference intervals for only a selection of serum biochemical analytes were established. The establishment of reference intervals for haematological and other various serum biochemical analytes is recommended.

Conclusion

This is the first report of baseline reference intervals for serum biochemical analytes of healthy captive and free-ranging cheetahs in Namibia based on the guidelines stipulated by the IFCC and CLSI regarding the generation of reference intervals for veterinary species. These reference intervals can be considered a useful tool for veterinary practitioners, researchers, zoologists and any other profession working in cheetah medicine and conservation. These results will aid in the accurate assessment of health and in the management of disease in cheetahs. Further research into the pathophysiology of diseases affecting cheetahs, using comprehensive health assessment data, is required to fully understand the threats facing this vulnerable species.

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Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Authors' contributions

G.C.H.L. (University of Pretoria) was responsible for acquisition of all data, performed statistical analyses of the data, interpretation of data and writing the manuscript. J.P.S. (University of Pretoria) made conceptual contributions to the protocol and manuscript. E.H.H. (University of Pretoria) assisted with statistical

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analyses and made conceptual contributions to the manuscript. SKH (IZW) collected the samples of the free-ranging cheetahs and made contributions to the manuscript. A.S.W.T. (NZG) was the project leader and responsible for conception and design of the project, collection of samples and made conceptual contributions to the protocol and manuscript.

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