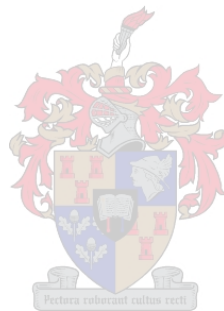# The effects of a *Mycobacterium bovis* infection on the metabolic and reproductive systems of African lions (*Panthera leo*) in the Kruger National Park.

Ignatius Michael Viljoen

Dissertation presented for the degree of Doctor of Philosophy (Molecular Biology) in the Faculty of Medicine and Health Sciences at Stellenbosch University.

Supervisors: Prof. P D van Helden (Stellenbosch University)
              Prof. R P Millar (University of Pretoria)

March 2017

Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This Dissertation includes one original paper published in a peer reviewed journal. The development and writing of the paper were the principal responsibility of myself. A declaration is included in the dissertation indicating the nature and extend of the contributions of co-authors to said publication.

Date: March 2017

Abstract

Lions (*Panthera leo*) are classified as vulnerable under the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, and lion populations are at risk owing to anthropogenic threats and infectious disease, amongst many factors. Tuberculosis (TB) caused by *Mycobacterium bovis* has the potential to pose a threat to wild lion populations in areas where it is endemic. TB in Kruger National Park (KNP) lions was first reported in the early 1990's and can lead ultimately to death of infected individuals. Despite obvious mortality of individual lions and changes in prides owing to deaths, consensus has not been reached on whether TB will negatively impact lion populations and resultantly there are currently no interventions in place to manage TB in KNP lions. Factors contributing to the lack of evidence based intervention, are a lack of definitive antemortem diagnostic tests and a lack scientifically sound knowledge on infection, disease progression, and ultimate effects of disease on individual lions.

This thesis serves as an attempt to start filling some of the above mentioned knowledge gaps. The introduction is a comprehensive review of what is known of TB in lions. From this it was possible to identify areas that are in need of further investigation. The rest of this thesis investigates the possible effects that *M. bovis* might have on lions' energy metabolism, immune/inflammatory response, and reproductive endocrinology.

In order to investigate these metabolic systems, proper diagnosis of diseased or infected lions was necessary. This study showed that the available diagnostic tests are in many instances lacking the necessary specificity for proper diagnosis of *M. bovis* infections in captive lions. Regardless of the difficulties with diagnosis of TB in the lions used for current study, it was possible to show that the lions in the KNP (exposed to *M. bovis*) compared to captive (unexposed) lions were experiencing an immune/inflammatory response, differences were observed for energy metabolism biomarkers, and wild male lions had reduced testosterone production. It is speculated that these differences are due to the presence of *M. bovis* in the KNP lions, however, direct causal links could not be established in the current study. The study on the reproductive endocrine system showed that it was possible to make use of a provocative kisspeptin challenge test to investigate the neuro-endocrine functions of lions. The current study also showed the value of doing simultaneous multiple system investigations, since results obtained from the individual systems were often confirmed or given more relevance when viewed in the context of results obtained from the other systems. This study serves as an indication that *M. bovis* is most likely contributing to multiple metabolic system alterations in KNP lions that could be considered a threat to that lion population. However, more research in larger numbers of animals controlling for confounding variables will be needed to confirm or reject this hypothesis.

Opsomming

Leeus (*Panthera leo*) word volgens die Internasionale Unie vir die Bewaring van die Natuur (IUCN) se "Red List of Threatened Species" as kwesbaar geklassifiseer, aangesien hulle onder andere onderhewig is aan bedreigings deur die optrede van mense, asook deur infeksies met mens of dier as vektor. Tuberkulose (TB), veroorsaak deur *Mycobacterium bovis,* het die vermoë om wilde leeupopulasies te bedreig in gebiede waar dit endemies is. Die eerste gevalle van TB onder leeus in die Krugerwildtuin (KNP) is in die vroeë 1990's opgeteken en dit kan uiteindelik tot die dood van geïnfekteerde leeus lei. Ten spyte van die ooglopende dood van siek leeus en die veranderinge in die dinamika van leeutroppe wat dit meebring, heers daar nie konsensus oor die vraag of TB die leeupopulasie negatief sal affekteer nie en gevolglik bestaan daar tans geen ingrypingsmaatreëls nie. Faktore wat tot hierdie gebrek aan bewysgebaseerde tussentrede bydra, sluit in onsekerheid oor voordoodse diagnostiese toetse, asook 'n gebrek aan wetenskaplike kennis oor die aanvanklike besmetting, siekteverloop en die uiteinde van die siektestoestand by individuele leeus nie.

Hierdie verhandeling dien as 'n bydrae om voorgenoemde gebrek aan kennis en insig te begin aanspreek. Die inleiding is 'n omvattende oorsig van die beskikbare inligting oor TB in leeus. Op grond daarvan kon leemtes geïdentifiseer word wat verdere navorsing uitlig. In die finale instansie ondersoek die verhandeling die moontlike invloed van *M. bovis* op leeus se energiemetabolisme, immuun- en inflammatoriese reaksies, asook hul voortplantingsendokrinologie.

Om die metabolisme te ondersoek, is die korrekte diagnose van geïnfekteerde of siek leeus noodsaaklik. Die studie het bevind dat die beskikbare diagnostiese toetse, in baie gevalle nie spesifiek genoeg is vir die werklike diagnose van *M. bovis* -infeksies in leeus in aanhouding nie. Ongeag die probleme ervaar met die korrekte diagnose van leeus wat ingesluit was in die studie, was dit steeds moontlik om aan te toon dat leeus in die Krugerwildtuin (blootgestel aan *M. bovis*) in vergelyking met leeus in aanhouding (nie blootgestel nie) 'n immuunreaksie toon, dat daar verskille was tussen die energiemetabolisme se biologiese merkers en dat wilde mannetjieleeus verlaagde testosteroonvlakke het. Die verskille is vermoedelik te wyte aan die teenwoordigheid van *M. bovis* in die Krugerwildtuinleeus, hoewel oorsaaklike verband nie in die huidige studie vasgestel kon word nie. Die ondersoek van die voorplantingsendokrinologie het getoon dat dit moontlik is om met behulp van 'n uitdagingstoets met kisspeptien as provokasiemiddel, leeus se neuro-endokriene funksies te ondersoek. Die huidige studie het ook die waarde van gelyktydige ondersoeke van verskeie stelsels uitgelig, aangesien resultate verkry van een sisteem op 'n gereëlde basis bevestig is of meer insiggewend was wanneer dit vergelyk word met die ander stelsels se resultate. Die verhandeling dui op die moontlikheid dat *M. bovis* waarskynlik bydra tot veranderinge in verskeie metaboliese stelsels in Krugerwildtuinleeus wat moontlik 'n bedreiging vir daardie populasie kan wees. Voortgesette navorsing op 'n groter aantal leeus, terwyl kontrole uitgeoefen word oor strengelveranderlikes om die hipotese te bevestig of te verwerp, word benodig.

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- Professor Paul van Helden at the Division of Molecular Biology and Human Genetics, Stellenbosch University, for his supervision over the study. This included, but was not limited to, help in obtaining funding for the project, putting me in contact with various role players, assistance in proof reading and editing of the thesis, and guidance in interpretation of the results.

- Professor Robert Millar at the Mammal Research Institute, University of Pretoria, for his supervision over the study. This included, but was not limited to, putting me in contact with various role players, assistance in proof reading and editing of the thesis, supplying of office space and laboratory space and equipment, and guidance in interpretation of the results.

- Various people from the Kruger National Park (KNP), SANParks, played instrumental roles in aiding me with the gathering of samples, organising accommodation, making available laboratory facilities, and overall facilitation of working in the KNP. I would therefore like to thank Dr. Sam Ferreira and Dr. Danny Govender from Scientific Services at Skukuza; Dr. Markus Hofmeyr, Dr. Peter Buss, Johan Malan, Dr. Michele Miller, Jenny Hofmeyr (Joubert) and all the staff of the Veterinary Wildlife Services at Skukuza; Marius Kruger and the rest of the KNP Veterinary Wildlife Service's wildlife capture team.

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List of Abbreviations

A

ACTH	adrenocorticotrophin
AD	adult
ARC	arcuate nucleus
AUC	area under the curve

B

BAL	bronchoalveolar lavage
BCG	bacillus-Calmette-Guerin
BL	body length: Used in calculating BMI of lions, measured in meters from the occiput on the back of the head to the base of tail (at point of indentation when tail is lifted vertically)
BMI	Body Mass Index, calculated for lions with the formula BMI = (1/(BL x SH)) x mass

C

CCI	(cortisol/CRP) x IP-10
CMI	cell-mediated immune responses
CNS	central nervous system
CRP	C-reactive protein

D

DM	diabetes mellitus
DPP	Dual Path Platform

E

EAG	estimated average glucose
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay

F

FIV	feline immunodeficiency virus
FIVple	Lion specific FIV strain
FSH	follicle-stimulating hormone

G

GEA	gene expression assay
GnIH	gonadotropin-inhibotory hormone
GnRH	gonadotropin-releasing hormone

H

HbA1c	Haemoglobin A1c / glycated haemoglobin
HiP	Hluhluwe-iMfolozi Park
HOMA-IR	homeostasis model assessment of insulin resistance

| HPA | hypothalamic-pituitary-adrenal |
| HPG | hypothalamic-pituitary-gonadal |

I

I	probably infected lion (in figures of cluster analyses dendogram)
ITT	intradermal tuberculin skin test
IUCN	International Union for Conservation of Nature
IV	intra venous

J

| JA | young adult |
| JU | juvenile |

K

KNP	Kruger National Park
KP	Kisspeptin
Kp-10	Kisspeptin-10
KZN	KwaZulu-Natal

L

| LH | luteinizing hormone |

M

MAC	*M. avium* complex
MPG	mean plasma glucose
MTC	*Mycobacterium tuberculosis* complex
MW test	two-tailed unpaired Mann Whitney test

N

NHLS	National Health Laboratory Services
NTM	Non-tuberculous mycobacteria
NZG	National Zoological gardens in Pretoria

P

PBMC	peripheral blood mononuclear cells
PC-1	First principle component
PC-2	Second principle component
PCA	Principle component analyses
PP	prepubertal
PPD	purified protein derivative
PVT	privately owned lions

Q

QFT	QuantiFERON®-TB Gold tubes
QFT-NIL	QFT tube with no antigens
QFT-TB	QFT tube with MTC antigens

ΔQFT   background corrected cytokine data by subtracting results obtained in QFT-NIL tubes from results obtained in QFT-TB tubes)

R

R_0 reproductive rate

RFRP's   RFamide peptides (mammalian orthologs of avian GnIH)

RIA   Radioimmunoassay

ROC   receiver operator characteristic

S

SA   sub-adult

SAA   serum amyloid A

SH   shoulder height: Used in calculating the BMI of lions, measured in meters from centre of the metacarpal pad in a straight line to the top of ridge of the scapula (keeping the leg and measuring tape straight and not following the curve of the shoulder)

SOP   Standard operating procedure

T

TB   Tuberculosis

TH   helper T cells

U

U   probably uninfected lion (in figures of cluster analyses dendogram)

W

WB   whole blood

Welch test   two-tailed unpaired t-test with Welch's correction

Z

ZN   Ziehl-Neelsen

Chapter 1

Introduction and aims

Tuberculosis (TB) due to *Mycobacterium bovis* was first reported in captive African lions (*Panthera leo*) in the 1990's in a German zoo with subsequent reports coming from both captive and wild populations. Some publications focused on disease pathology and diagnosis in lions with a handful touching on the possible negative effects of the disease on lion populations. Despite proof that TB due to *M. bovis* ultimately can lead to morbidity and death of individual lions, consensus still needs to be reached on the implications of TB on the survival of wild lion populations. In my opinion a contributing factor to this lack of agreement between the different stakeholders is the inability to easily access comprehensive data on TB in lions. In order to address this and simultaneously to identify areas where more research is needed I undertook a comprehensive review of *M. bovis* infection in lions. This review was published in The Journal of Veterinary Microbiology and will also serve as the introductory chapter of this dissertation. For the purpose of this dissertation an unedited copy of the published review (in Microsoft$^{®}$ Word$^{®}$ format) is provided on pages three to 20.

1.1 Introduction (published review article)

The complete reference for the review article is:

Viljoen, I M, van Helden, P D, Millar, R P. 2015. *Mycobacterium bovis* infection in the lion (*Panthera leo*): current knowledge, conundrums and research challenges. Veterinary Microbiology. 177, 252-260.

Included on the following page is a declaration from all contributing authors with regards to their involvement in the writing of the manuscript.

Declaration of contribution to published article included in Chapter 1

Declaration by the candidate:

With regards to the published review article in Chapter 1 the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Literature review and writing of the article	90%

The following co-authors have contributed to the published review article:

Name	e-mail address	Nature of contribution	Extent of contribution (%)
Prof P D van Helden	PVH@sun.ac.za	Proof reading and advice with regards to general structure of manuscript, paragraphs and sentences. Advice on literature to include or exclude.	5%
Prof R P Millar	robertpetermillar@gmail.com	Proof reading and advice with regards to general structure of manuscript, paragraphs and sentences. Advice on literature to include or exclude.	5%

Signature[1] of candidate: _____ Date: _____

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to the published review article in Chapter 1.

2. no other authors contributed to the published review article in Chapter 1 besides those specified above, and

3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 1 of this dissertation.

Name	Signature[1]	Institutional affiliation	Date
Prof P D van Helden		Stellenbosch University	
Prof R P Millar		University of Pretoria	

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[1] Declaration with signatures in possession of candidate and supervisor

Mycobacterium bovis infection in the lion (Panthera leo): current knowledge, conundrums and research challenges.

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Abstract

Mycobacterium bovis has global public-health and socio-economic significance and can infect a wide range of species including the lion (*Panthera leo*) resulting in tuberculosis. Lions are classified as vulnerable under the IUCN Red List of Threatened Species and have experienced a 30% population decline in the past two decades. However, no attempt has been made to collate and critically evaluate the available knowledge of *M. bovis* infections in lions and potential effects on population. In this review we set out to redress this. Arguments suggesting that ingestion of infected prey animals are the main route of infection for lions have not been scientifically proven and research is needed into other possible sources and routes of infection. The paucity of knowledge on host susceptibility, transmission directions and therefore host status, manifestation of pathology, and epidemiology of the disease in lions also needs to be addressed. Advances have been made in diagnosing the presence of *M. bovis* in lions. However, these diagnostic tests are unable to differentiate between exposure, presence of infection, or stage of disease. Furthermore, there are contradictory reports on the effects of *M. bovis* on lion populations with more data needed on disease dynamics versus the lion population's reproductive dynamics. Knowledge on disease effects on the lion reproduction and how additional stressors such as drought or co-morbidities may interact with tuberculosis is also lacking. Filling these knowledge gaps will contribute to the understanding of mycobacterial infections and disease in captive and wild lions and assist in lion conservation endeavours.

Keywords: Bovine Tuberculosis; *Mycobacterium bovis; Panthera leo;* Lion; Mycobacterial disease; Wildlife conservation.

1. Introduction

Mycobacterium bovis forms part of the pathogenic *Mycobacterium tuberculosis* complex group of organisms (Brosch et al. 2002). Its ability to infect a wide range of livestock and wildlife species, as well as humans, highlights its global public-health and socio-economic significance

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(Ayele et al. 2004; Michel et al. 2006; Renwick et al. 2007; OIE, 2012). Additionally, *M. bovis* can be considered an invasive species in ecosystems where it historically did not occur (Michel et al. 2006; Ferreira & Funston, 2010). In 1929 a report on the presence of *M. bovis* in greater kudu (*Tragelaphus stresiceros*) and other small ungulates in the Eastern Cape Province of South Africa suggested a potential transmission of *M. bovis* from domestic cattle to African wildlife species (Michel et al. 2006; OIE, 2012). De Vos et al. (2001) stated that *M. bovis* showed all indications of causing ecological imbalance in the Kruger National Park (KNP) ecosystem and had at that stage already taken on epidemic proportions.

Since 1929, other African wildlife species reported to have been infected with *M. bovis* include African buffalo (*Syncerus caffer*), wildebeest (*Connochaetes taurinus*), bushpig (*Potamochoerus porcus*), chacma baboon (*Papio cynocephalus*), cheetah (*Acinonyx jubatus*), common duiker (Sylvicapra *grimmia*), eland (*Taurotragus oryx*), honey badger (*Mellivora capensis*), impala (*Aepyceros melampus*), large spotted genet (*Genetta tigrina*), leopard (*Panthera pardus*), lechwe (*Kobus leche*), lion (*Panthera leo*), spotted hyaena (*Crocuta crocuta*), and warthog (*Phacochoerus aethiopicus*) (Keet et al. 1996; de Vos et al. 2001; Cleaveland et al. 2005; Michel et al. 2006; Trinkel et al. 2011; OIE, 2012). All species do not appear to have the same susceptibility to infection with *M. bovis* and their role in the epidemiology can be roughly grouped into spillover hosts and maintenance hosts (Ayele et al. 2004). In maintenance hosts, infection can persist in a population without reinfection events from other species. In contrast, spillover host populations need to be reinfected from other sources in order for the infection to persist. Cattle and other bovids are arguably the most well known maintenance hosts (Ayele et al. 2004).

African buffalo are a maintenance host of *M. bovis* in much of their range with *M. bovis* endemic in the buffalo populations of the KNP and the eastern KwaZulu-Natal (KZN) province of South Africa. *M. bovis* in hosts such as buffalo can potentially be transmitted to other susceptible species – including domestic cattle - that can either also serve as additional maintenance hosts or spillover hosts (de Vos et al. 2001; Renwick et al. 2007). The presence of *M. bovis* in certain lion populations has been ascribed to transmission from infected buffalo (Renwick et al. 2007; Michel et al. 2009).

Lions are apex predators in the African habitat with their presence or absence determining the survival of various other animals (carnivores and herbivores) which in turn can even affect the flora and overall biodiversity in a specific area. Lions are a major tourist attraction and thus economically important. Together with other species in the large predator guild they have the capacity to act as flagship or sentinel species for conservation efforts (Dalerum et al. 2008).

It is therefore important to establish the effect of *M. bovis* infections on lions in order to make informed decisions concerning their management and conservation. This review is aimed at summarising current publications of tuberculosis in lions, critically analysing them and identifying additional research required to allow informed policy and intervention strategies.

2. Overview of tuberculosis case reports in lion

The lion is classified as vulnerable under the IUCN Red List of Threatened Species and has experienced a 30% population decline over the past two decades (Nowell et al. 2012). This is primarily due to the killing of lions to protect human life and livestock as well as a reduction in wild prey availability and habitat loss. Additionally, disease is also considered a threat to lion populations (Nowell et al. 2012).

The first reported cases of lions contracting tuberculosis (TB) came from two different zoos (Eulenberger et al. 1992; Morris et al. 1996). In 1992, Eulenberger et al. reported on the cases of tuberculosis and the management thereof in primates and felids in the Leipzig Zoological Gardens in Germany from 1951-1990. Although this report focussed on all felid species housed at the zoo - including leopard (*Panthera pardus*), tiger (*Panthera tigris*), puma (*Puma concolor*), lynx (*Lynx lynx*) - the species most often diagnosed with TB was the lion (Eulenberger et al. 1992). The high level of infection recorded (12 cases in 39 years) was not regarded as an indication of lion susceptibility to TB, but rather ascribed to the manner in which the zoo lion population was housed. They did not specify the *Mycobacterium sp.* that infected the lions. However, of all the felids, seven of the TB cases were confirmed to be due to *M. bovis* infections, while 19 cases were undetermined. No cases of *M. tuberculosis* were reported (Eulenberger et al. 1992). The onset of disease was relatively sudden after the felids experienced high stress situations such as after repeated periods of pregnancy and lactation. Other signs of disease observed in the felids were a lack of movement associated with an overall loss of condition and body weight as well as severe dyspnoea (Eulenberger et al. 1992). The lungs were the main organ affected in felids, suggesting that the route of infection in these cases was through airborne droplets. Additionally, alterations in the intestine and intestinal lymph nodes suggested the possibility that infection could also have occurred through the ingestion of infected meat (Eulenberger et al. 1992).

The report from the second zoo was published in 1996 and reported on TB due to *M. bovis* in only lions. An eight year old male lion in the Knoxville Zoo, USA, was euthanised in 1985 due to its continued deteriorating health (Morris et al. 1996). The first signs that the lion was diseased were a three week history of weight loss and anorexia. Other clinical signs observed are listed under section 3 of this review. Diagnosis of *M. bovis* infection was demonstrated post mortem by isolation from a trachebronchial lymph node (Morris et al. 1996). This lion was in direct contact with a lioness and in indirect contact with two other younger lions. Three years after the diseased lion was euthanised, follow-up examinations were done on the remaining three lions with normal results obtained for physical examinations, whole blood counts and serum chemistry. Morris et al. (1996) were not able to establish the route of infection for the male lion. No mention was made concerning the age or origin of the infected lion at the time of procurement by the Knoxville Zoo. Considering that *M. bovis* can be latent for a long period of time in some animal species, infection could have occurred before arrival at the zoo.

The first report of *M. bovis* in free ranging African lions was in 1996 in two lionesses in the KNP, South Africa (Keet et al. 1996). These two lionesses were both approximately 10 years of age with one emaciated to the degree that she could hardly stand. Both of the females had lung lesions that were morphologically similar. Acid-fast bacilli were detected in smears made from the exudate of these lesions and culture confirmed the presence of *M. bovis* (Keet et al. 1996). Since this report many more lions with tuberculosis have been identified in the KNP (Keet et al. 2000; Keet et al. 2010). Most of the confirmed tuberculosis cases in lion came from the central and southern regions of KNP, corresponding with the regions of high *M. bovis* prevalence in buffalo herds (Renwick et al. 2007). Keet et al. (1996), while referring to the report of Eulenberger et al. (1992), proposed that the most likely route of exposure was via the alimentary route from eating contaminated buffalo carcasses. Many diseased lions have since been euthanised and generated considerable data (Kirberger et al. 2006; Keet et al. 2010; Trinkel et al. 2011) (see details in later sections).

The presence of *M. bovis* in wildlife in general has also been reported in KZN with confirmation of lions being infected in the Munyawana Game Reserve (Michel et al. 2009) and the Hluhluwe-iMfolozi Park (HiP) (Michel et al. 2006; Michel et al. 2009; Trinkel et al. 2011). In HiP, infection with *M. bovis* was confirmed from post mortem inspection and culture of samples from lions that had died naturally (presumably from tuberculosis) or that were euthanised due to advanced emaciation (Trinkel et al. 2011). The main route of exposure for HiP lions was believed to be ingestion of contaminated buffalo carcasses (Trinkel et al. 2011). Unfortunately, disease pathology was not described in this study.

Elsewhere in Africa *M. bovis* was reported in free ranging lions in the Serengeti National Park, Tanzania (Cleaveland et al. 2005). Lion blood serum samples collected over the period 1984 to 2000 were subjected to *M. bovis* antibody enzyme immunoassay (EIA). The serological results suggested that mycobacterial infection was present in the Serengeti lions from as early as 1984. Although the serology results could not identify the species of the *Mycobacterium tuberculosis* complex involved, isolation of *M. bovis* from lion prey species suggested it as a likely candidate (Cleaveland et al. 2005).

3. Clinical signs and physiological changes accompanying *M. bovis* infection in lions

The degree to which lion health is impacted by tuberculosis is largely unknown. Progress of tuberculosis in lions is apparently slow, with the majority of infected lions appearing healthy while being sub-clinically infected (Keet et al. 2010). Unfortunately, clinical signs of active disease appear only when the disease has progressed to an advanced stage. Antemortem clinical signs associated with progressive tuberculosis are: marked alopecia and old, poorly healed bite wounds (Keet et al. 1996; Keet et al. 2000), emaciation (Morris et al. 1996; Keet et al. 1996; Keet et al. 2010; Trinkel et al. 2011), corneal opacity (Keet et al. 1996; Keet et al. 2000); dyspnoea and tachypnoea (Eulenberger et al. 1992; Morris et al. 1996; Cleaveland et al. 2005), bilateral sub-mandibular swelling (Cleaveland et al. 2005), ataxia and hypermetria (Cleaveland et al. 2005) and

bilateral pulmonary disease (observed by means of thoracic radiography) (Morris et al. 1996). Cytology of bronchoscopic aspirate revealed pyogranulomatous exudates with many macrophages, moderate numbers of mature non-degenerate neutrophils, and a few plasma cells and lymphocytes (Morris et al. 1996). Abnormal whole blood counts included leukocytosis with a mature neutrophilia and slight toxic granulation, and monocytosis, while serum chemistry abnormalities included hypoalbuminaemia, hyperglobulinaemia, and hypercalcaemia (Morris et al. 1996). Keet et al. (2000) reported similar haematology and blood chemistry findings and suggested that *M. bovis* in lions causes haematological changes similar to that seen in alimentary tract infections associated with malabsorption (Keet et al. 2000). In humans there is a strong association between active tuberculosis and diabetes mellitus (DM) with indications that DM is a significant risk factor for developing active TB and/or vice versa (Broxmeyer, 2005; Harries et al. 2009; Mao et al. 2011; Gupta et al. 2011). Human TB is associated with altered energy metabolism and homeostasis in patients with active TB (Broxmeyer, 2005; Bell et al. 2007; Bottasso et al. 2010; Santucci et al. 2011) that could actually aid in the development of type-2 diabetes (Broxmeyer, 2005). Whether or not such associations exist in lions is unknown.

Tuberculous lesions in lions differed macroscopically from that described in ungulates and non-human primates (Keet et al. 2000; Renwick et al. 2007; Keet et al. 2009). Only pulmonary lesions were macroscopically diagnostic while all other lesions were difficult if not impossible to identify macroscopically (Keet et al. 2000).

The lesions observed in various organs were granulomatous, typical of tuberculosis lesions in other species. Histologically they consist of macrophages, epithelioid cells, lymphoplasma cells and neutrophils (Keet et al. 2010). In addition lung and lymph node lesions showed extensive fibrosis and scant focal necrosis. Bronchiectasis and exudative tuberculous bronchitis was also observed in the lungs (Keet et al. 2000). Table 1 summarises the lesion characteristics and the macroscopic and microscopic pathology associated with the various organ systems.

Since there are over 130 known species of Mycobacteria, caution is required to not over diagnose *Mycobacterium tuberculosis* complex by simple lesion observation or smear testing (Botha et al. 2013). Acid-fast bacilli were often sparse or absent from histological sections, even from some culture-positive cases (Keet et al. 2010). Non-tuberculous mycobacteria (NTM) were frequently cultured from lions and might have been responsible for the observation of acid-fast bacteria in suspicious and microscopic lesions (Keet et al. 2010). As a result, Keet et al. (2010) did not use histology to enhance the specificity and/or sensitivity of their culture gold standard while validating the lion intradermal tuberculin skin test (Keet et al. 2010).

Table 1**:** Tuberculosis lesions: characteristics and micro- and macro-pathology associated with the different lion organ systems.

Organ or System	Lesions	References
Respiratory system	More often associated with advanced cases of tuberculosis; Consolidation with ill-defined lesions, sometimes confluent, firm, pliable foci approximately 4 cm in diameter; Varying levels of acid-fast bacilli. Pneumonia consisted of an amorphous, multifocal to coalescing, expansile (non-encapsulated) granulomatous inflammatory reaction without necrosis, giant cells or calcification;	Keet et al. (1996), Keet et al. (2000), Keet et al. (2010)
Intestine	Miliary distributed, microscopic, lesions with small granulomas in submucosa; Mononuclear macrophage predominance suggestive of mycobacterial mural enteritis.	Keet et al. (2000), Keet et al. (2010)
Skeletal	Granulomatous osteitis, periostitis and osteosis, frequently associated with myositis; *M. bovis* induced osseous lesions were more likely to involve the joints; Proliferative septic arthritis, joint capsule mineralization, and bone slivers may be good indicators of *M. bovis* infection; Unilateral lesions in the proximal tibia (more often seen in adult males) and tibio-tarsal joints, the proximal radius and ulna, and the thoracic vertebrae with various degrees of hind limb atrophy and lameness; Numerous intracellular acid-fast organisms present in elbow joint hygromas in adult lions; Elbow hygromas more frequently seen in females.	Keet et al. (2000), Keet et al. (2009), Keet et al. (2010), Kirberger et al. (2006),
Lymphatic system	Visible, palpable and marked enlargement of superficial lymph nodes not present in lions; Miliary distribution of lesions in nodes in early stage infection; Marked lymphoid atrophy with no evidence of tuberculosis; Generalised lymphadenopathy accompanied by cystic dilation in the paracortical areas observed after necropsy; Lesions present in many of the lymph node groups, regardless of degree of infection; Granulomatous lesions containing eosinophils and parasitic remnants observed.	Keet et al. (1996), Keet et al. (2000), Keet et al. (2009), Keet et al. (2010)
Eyes	Granulomatous panophthalmitis, choroiditis, uveitis, and conjunctivitis, with or without retinal detachment.	Keet et al. (2000)
Kidneys	Amyloidosis in the medulla being more severe in advanced tuberculosis cases.	Keet et al. (1996), Keet et al. (2000)
Other	Microscopic lesions in liver and bone marrow; Non-specific muscle atrophy accompanied by cachexia, decubitus ulcers; Testis atrophy; None of 86 necropsied females were pregnant.	Keet et al. (2000), Keet et al. (2009)

4. Routes of exposure

Domestic cats may be infected with *M. bovis* from infected food sources (Little et al. 1982; Morris et al. 1996). Eulenberger et al. (1992) were the first to suggest that lions could be infected with *Mycobacterium sp.* by ingesting infected meat. They also suggested horizontal transmission between lions by means of aerosol droplets (Eulenberger et al. 1992). Hence, Keet et al. (1996) suggested that wild lions become infected with *M. bovis* firstly through consumption of infected prey after which spread of the disease to other pride members could occur via droplet transmission. Additionally, the possibility also exists that lions can be exposed to *M. bovis* while suffocating prey (e.g. African buffalo) by biting over the muzzle of the prey (Renwick et al. 2007). The finding that lions are able to shed viable *M. bovis* through the respiratory system argues for droplet transmission (Miller et al. 2015). However, definitive evidence for these routes of infection is lacking. Other routes of transmission may also be involved, for example percutaneous infection occurring during social aggression, to explain hygromas.

The epidemiology of tuberculosis in lions may differ between areas. In the KNP and HiP genotyping of *M. bovis* isolated from buffaloes and lions showed matching sequences although the sequences in the two areas were not the same. This suggested that *M. bovis* infection in these lions was due to transmission from infected buffaloes (Renwick et al. 2007; Michel et al. 2009).It was also considered that lion behaviour patterns (preferential prey species, individual prey selection, scavenging, and intra-species specific aggression) led to the frequent exposure of lions to *M. bovis* from different and varied species (Keet et al. 2000; Renwick et al. 2007). It is now commonly accepted that the principle route of infection for lions is through consumption of infected prey (Keet et al. 2000; Cleaveland et al. 2005; Kirberger et al. 2006; Keet et al. 2010). In this regard, Renwick et al. (2007) raised the concern that if *M. bovis* was to become established in additional prey species with maintenance host capabilities, the opportunity for contact with infectious prey would increase. Indeed the main suspected source of *M. bovis* infection for Serengeti lions was considered to be wildebeest and not the buffalo (Cleaveland et al. 2005).

That lions are a maintenance host has not been conclusively established. Lesions in many different sites have been described in infected lions, suggesting the possibility of several different routes of infection (Keet et al. 2000). Interestingly Renwick et al. (2007) stated that lions do not seem able to maintain *M. bovis* infection in the absence of infected maintenance hosts in an ecosystem. This suggests that lions may be end hosts (Renwick et al. 2007) in which there is little or no chance of onward transmission occurring. Anecdotal evidence for this comes from Morris et al. (1996), who described an infected male lion housed with a lioness who did not contract the disease. Serum samples from this lioness subjected to ELISA tests showed a decline in reactivity to both *M. bovis* and *M. avium* antigens at progressively longer time intervals after the male was euthanised (Morris et al. 1996). This could indicate that although the lioness was exposed to *M. bovis* her immune system managed to prevent progression to a diseased state. To our knowledge no other studies have provided further evidence for changes in reactivity over time.

5. *M. bovis* diagnostics for lions

The diagnosis of *M. bovis* infection in lions may involve gross post mortem examination with associated histopathology, bacteriological examination of clinical and post mortem samples, and immunological assays. The development and application of diagnostics for M. bovis infection is dependent on a number of factors. This includes whether the target is a living or dead animal, the degree of false positive and false negative findings for the test, test sensitivity, the practicality for field application and expense. The accepted gold standard for diagnosis of mycobacterial infection is still culture based (generally from tissues obtained at necropsy). This remains the benchmark for the validation of other diagnostic protocols or algorithms combining several tests. It should be mentioned that based on cattle studies the sensitivity of culture diagnosis of *M. bovis* is reliant on the technique and effort employed during post mortem examination and sampling of carcases (Corner, 1994). The first reported infections of *M. bovis* in wild lions employed culture of samples obtained from lungs and identification by standard bacteriological methods (Keet et al. 1996). *M. bovis* has also been isolated from the tracheobronchial lymph node (Morris et al. 1996).

Another option to obtain samples for culture is through bronchoalveolar lavage (BAL). Miller et al. (2015) managed to identify *M. bovis* infection in 6% of 134 lions tested in the KNP. However, this method can produce significant false negative findings. Aspiration liquid does not reach all areas of the lung and therefore can fail to recover viable organisms for culture (Somu et al. 1995; Miller et al. 2015) in infected lions. Additionally, processing (freeze-thaw) and transportation might also decrease viability of the samples. Therefore prevalence of shedding of *M. bovis* is likely to be higher in the KNP population (Miller et al. 2015). Infection in other organs or lymph nodes cannot be reached through BAL sampling, and sampling of these tissues requires biopsy (e.g. fine needle aspirate) which can be effective for some nodes. To our knowledge other clinical samples such as urine, faeces, saliva and nasal mucus has not been investigated as sources of organisms for culture. Keet et al. (2010) highlighted the importance of attempting culture from all organ systems irrespective of observable macroscopic lesions. For lions this is particularly important since multiple organ systems can be affected while lesions, other than pulmonary ones, are not readily identifiable macroscopically (Keet et al. 2010). Kirberger et al. (2006) managed to culture *M. bovis* from affected joints and a hygroma. Interestingly, histological slides of microscopic lesions stained with Ziehl-Neelsen had a notable absence of acid-fast bacilli despite the lions being shown to be culture positive (Keet et al. 2010).

An option for antemortem diagnosis of *M. bovis* infection is the use of ELISA/EIA antibody tests using *M. bovis* and *M. avium* antigens. Morris et al. (1996) suggested that they could serve as a sensitive supplement to traditional diagnostic tests. Morris et al. (1996) utilised ELISA tests on blood samples from an infected male lion, an in-contact lioness, and two juvenile lions indirectly in-contact with the male lion. Comparing the ELISA results to those obtained from nine healthy control lions showed that the diseased male had much higher antibody levels while the two juveniles had basal values. The in-contact female had intermediate levels of antibodies. The in-

contact female was sampled one and three years after the male was euthanised and showed a decrease in antibody levels. The lioness and the juveniles were still asymptomatic respectively five years and nine years after the diseased lion was euthanised (Morris et al. 1996). In future useful information on infection and progression to disease (or lack thereof) could be gained from serological tests repeated over a few years in infected and exposed lions. While this might be suitable for a captive lion study, repeat sampling of free roaming lions would be more difficult. A more, *M. bovis* specific once-off antemortem diagnosis would be more optimal for wildlife management. However, it needs to be recognised that the presence of *M. bovis* specific antibodies simply indicates that the animal has been exposed to *M. bovis* at some time and does not indicate current infection or active disease.

Two separate studies from the Serengeti National Park and the KNP utilised a similar serological test using MPB70 antigen (Cleaveland et al. 2005; Keet et al. 2010). MPB70 is thought to be highly specific to *M. bovis* and was initially purified from *M. bovis* bacillus-Calmette-Guerin (BCG) (Nagai et al. 1981; Harboe et al. 1990). Four percent of 184 Serengeti lion tested seropositive for tuberculosis. No cultures were done to confirm infection with *M. bovis* (Cleaveland et al. 2005). Since MPB70 is highly expressed by *M. bovis* (Wiker et al. 1998) it is reasonable to assume that the Serengeti lions were indeed exposed to *M. bovis*. However, there may be some level of non-specificity as Rhodes et al. (2011) showed antibody production to MPB70 in cats infected with *Mycobacterium* complex species other than *M. bovis* (Rhodes et al. 2011). Due to the limited sensitivity of the ELISA Cleaveland et al. (2005) stated that the 4% seroprevalence might suggest the minimum prevalence of *M. bovis* in the Serengeti lion population. In the KNP study only 12 of 26 confirmed *M. bovis* positive lions with advanced clinical disease gave seropositive results (Keet et al. 2010) also indicating low sensitivity.

The low sensitivity of the serological assays is not limited to lions. Serological assays, while in some instances specific, do not have the necessary sensitivity to serve as reliable antemortem diagnostic tests for individual animals (Harboe et al. 1990; de Lisle et al. 2002; Chambers et al. 2008). However, increased sensitivity of serological assays may be seen in animals with advanced tuberculosis (Harboe et al. 1990; de Lisle et al. 2002; Chambers et al. 2008). Use of serological tests in concert with cellular immune based diagnostic tests could increase the sensitivity of identifying *Mycobacterium* infected animals (Harboe et al. 1990; Gutiérrez et al. 1998; de Lisle et al. 2002). In most animal species a specific cell-mediated response is detectable following infection but in advanced cases cell-mediated immune responses may decline and high levels of antibody may become apparent (Harboe et al. 1990; de Lisle et al. 2002).

The lack of reliable serological or cell-mediated immune assay led Keet et al. (2010) to explore the use of the intradermal tuberculin skin test as an antemortem diagnostic test for *M. bovis* in lions. Although skin testing of free roaming wildlife species presents logistical difficulties, Keet et al. (2010) managed to show the inherent value of doing such diagnostic tests on lions. By modifying the method used to test domestic cattle, it was possible to identify over 86.5% of lions

(n=52) in which *M. bovis* infection was confirmed through mycobacterial culture. The two main alterations to the established cattle protocol were: the use of 0.2ml tuberculin per injection site (double the volume prescribed for cattle); and while both avian and bovine tuberculin were injected at separate sites, they only considered the result of the bovine tuberculin reaction (Keet et al. 2010). However, 13.5% of culture positive lions tested negative (false negative) and 18.8% of true negative animals tested positive (false positive) (Keet et al. 2010). Additionally, skin testing of free ranging lions is a logistical challenge as it requires holding the animals for or recapturing them after 72 hours to record a response.

As discussed above, assays of the cell-mediated and humoral immune responses could be evaluated in parallel to increase the accuracy of diagnosis. Keet et al. (2010) suggested that serological diagnostics could serve to compliment the intradermal tuberculin test, specifically in cases of non-reactor lions with advanced tuberculosis. However, the progression of *M. bovis* infection could be accelerated by feline immunodeficiency virus (FIV) co-infection. However, co-infection could ultimately also lead to reduced antibody levels (Keet et al. 2010) thereby negatively affecting serological assays. If this is the case it could also help to explain the low *M. bovis* seroprevalence (4%) described for Serengeti lions by Cleaveland et al. (2005) (See Section 7 for FIV prevalence).

Another test that is at least as sensitive as the skin test in other species, but with the ability to detect infection marginally earlier, is the IFN-γ test that also relies on the CMI (de la Rua-Domenech et al. 2006). Unfortunately this test does not distinguish between infection status (e.g. recent, latent or advanced/diseased) (Pal et al. 2008) and can be affected by diverse pathophysiological and physiological events. Maas et al. (2010) described the genetic sequence of lion IFN-γ. They compared it with the cheetah and domestic cat sequences and found that the sequences are highly conserved between these species. They suggested the possibility that a lion or cat specific IFN-γ ELISA if developed could be used for other feline species (Maas et al. 2010). Further investigations to establish cross reactivity with existing IFN-γ tests or one developed with cat IFN-γ antibodies is also needed (Maas et al. 2010). In humans, co-morbidities with parasites and some viral infections as well as iron deficiencies and even younger age can increase the likelihood of an indeterminate IFN-γ assay results (Banfield et al. 2012). This might have implications for lions with co-morbidities of other pathogens such as FIV or canine distemper virus, thus raising doubts on the sensitivity of the test.

6. Effect of *M. bovis* on lion populations

Keet et al. (1996) raised the concern that due to the interactions of lions, the disease could become established in the KNP lion population. If not already the case, lions might then serve as an additional maintenance host for *M. bovis* together with the buffalo. From the available literature it is clear that lions are spillover hosts but it is unclear if lions are end or maintenance hosts (Keet et al. 1996; Michel et al. 2006; Renwick et al. 2007; Michel et al. 2009) (see earlier). Interestingly disease characteristics determined for buffalo also appear to be manifested in lions (Michel et al.

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2006). These include mortality due to disease, correlations between age and infection, as well as correlations between infection and body condition (Michel et al. 2006).

There may be multiple behavioural and social effects of infection within an infected population. The lameness caused by a *M. bovis* infection might impact on hunting success of free ranging lions (Kirberger et al. 2006) or compromise the ability of an individual to compete in a natural environment. Michel et al. (2006) speculated that *M. bovis* was driving social changes within prides, contributing to lower lion survival and breeding success. This included faster territorial male coalition turnover with consequent infanticide, and the eviction of entire male and female prides from territories (Michel et al. 2006; Keet et al. 2009). A comparison between infected and non-infected sub-populations showed that the non-infected sub-population was significantly longer lived and had a higher cub survival rate even though the infected sub-population had a higher birth rate (Michel et al. 2006; Keet et al. 2009).

Due to the social nature of lions it is possible for TB compromised individuals to retain support from the rest of the pride. This gives the affected individual a better chance of surviving than would be the case for solitary animals. Estimated time from infection to death is between two and five years (Renwick et al. 2007). While most of the lions that died as a consequence of tuberculosis were older than five years, some reports also included younger lions, the youngest euthanised being 16 months old (Morris et al. 1996; Keet et al. 1996; Cleaveland et al. 2005; Kirberger et al. 2006; Trinkel et al. 2011). However, currently there is insufficient data to make definitive statements concerning the average age lions may reach when experiencing active disease. There is also no data on indirect mortalities due to *M. bovis*, such as cub mortalities owing to inadequate parental care from diseased adults.

Environmental factors may also influence how tuberculosis affects lion populations. Cleaveland et al. (2005) did not report on actual effects of *M. bovis* infection on the Serengeti lion population. They did, however, speculate that conservation practices might have an effect on how tuberculosis establishes in wildlife species and how the wildlife adapts to a new disease. In South Africa *M. bovis* is considered a relatively new pathogen introduced to naïve wildlife populations. Stringent control measures in livestock farming and the fact that South African wildlife and livestock are usually separated by fences may have limited the transmission of *M. bovis* to wildlife (Cleaveland et al. 2005). In contrast, Cleaveland et al. (2005) suggested that the continued practice of fenceless husbandry in East Africa might have permitted the spread and establishment of *M. bovis* infection in wildlife over a longer time period, perhaps even permitting a more stable endemic pattern of infection.

Ferreira and Funston (2010) disputed the conventional wisdom of how disease can influence carnivore populations. They suggested that *M. bovis* in prey appears to have little detectable influence on lion demography in KNP while prey biomass is of more importance. Additionally, differences in lion body condition scores were apparently not associated with *M. bovis* prevalence in prey (Ferreira & Funston, 2010). They were concerned, however, that the situation

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might change under different circumstances. Additionally, they pointed out some of the biases that could have affected their results. This included:

The assumption that lion population vital rates were stationary within the study zones for some time before the survey started.

That they assessed the relative importance of only two variables, namely prey biomass and *M. bovis* prevalence in prey. Therefore they could not separate the interactions in the northern part of KNP where they only had low disease prevalence, compared to the south where they had both medium and high prevalence.

The survey was done in a relatively wet period with high quality prey biomass. This is important since during wet periods KNP lions predate mostly on wildebeest and zebra (*Equus quagga)* and to a lesser extent on buffalo. Prey switching occurs during dry periods when the importance of buffalo as prey increases. Therefore, under drought conditions lions are more likely to come into contact with *M. bovis* infected prey (Ferreira & Funston, 2010).

In single host systems each pathogen and species of animal infected has a specific threshold population density that would allow a pathogen to infect and then be maintained in a population (Renwick et al. 2007). Determining persistence of pathogens in multiple host systems is more complicated with community thresholds changing in accordance to inter- and intra-species interactions (Renwick et al. 2007). Additionally persistence of a disease in a population is also influenced by the reproductive rate (R_0) of a pathogen. For a pathogen to survive indefinitely R_0 must be ≥ 1. Diseases can, however, persist and be a continued threat for significant periods when $R_0 < 1$, that is during the extinction phase, especially for chronic infections (Renwick et al. 2007). *M. bovis* is considered to be such a pathogen (Renwick et al. 2007).

Infection thresholds and disease dynamics have not been described for lions, therefore, we currently have very little idea concerning the ultimate effect of *M. bovis* on lion populations. There is no data available to show if disease dynamics is faster or slower than the lion's reproductive population dynamics. The definition or scale of population one adheres will also influence the results when interrogating population effects of tuberculosis and might be different from conservation area to conservation area. Even within large conservation areas like the KNP, quantifying disease versus population dynamics and community thresholds could be complicated by the variation in lion density in different regional zones. Keet et al. (2009) attempted some quantification for the KNP lion population. However, much of the data used in the model were derived from species other than lion and expert opinion.

Although Ferreira & Funston (2010) suggest that the persistence of lions in KNP is not threatened, they concede that the situation might change under different climatic conditions and in the presence of additional stressors. While it has been reported that *M. bovis* prevalence in prey does not have a negative influence on lion demography (Ferreira & Funston, 2010), *M. bovis* infection in lions definitely affects individual lions and can therefore also affect pride dynamics.

Should the lion population decline, inbreeding may become a problem with potentially serious consequences. In the HiP it was believed that the initial inbred status of the lion population in concert with tuberculosis had a bigger effect on the population with the effect subsequently being reduced when genetic diversity was introduced into the lion population (Trinkel et al. 2011).

7. FIV and *M. bovis* co-morbidities

Other diseases can have an effect on or be affected by the presence of an infectious disease such as tuberculosis. Feline immunodeficiency virus (FIV) and more specifically the lion specific FIV strain (FIVple) is thought to be a relatively old disease in lions (Roelke et al. 2009). FIV has been shown to cause CD4+ immune cell depletion in lions (Roelke et al. 2006), cells that, at least in cattle, are significant producers of IFN-γ that plays an important role in the host's immune response (Roelke et al. 2006). Neurological effects and wasting have been associated with FIV infection in captive lions (Roelke et al. 2009). In many instances increased cases of wasting were, associated with the outbreak of diseases such as tuberculosis or canine distemper virus with immunosuppressive effects (Roelke et al. 2009). Interestingly, some of the non-specific FIVple blood based abnormalities (Roelke et al. 2009) were similar to some of the non-specific *M. bovis* blood based abnormalities of a FIVple negative lion (Morris et al. 1996).

Some attempts have been made to determine if an FIV infection predisposes or exacerbates *M. bovis* infection. Keet et al. (1996) observed a high seroprevalence (83%) of FIV in the KNP lions who readily were infected with tuberculosis and that may have increased the susceptibility of lions to *M. bovis* infection.   On the other hand, studies by Cleaveland et al. (2005) and Kirberger et al. (2006) described a low number of lions with *M. bovis*-FIV co-infection and suggested that FIV seemingly has no influence on *M. bovis* infection in lions. These suggestions are, however, called in to question if one considers the low sensitivity of the tuberculosis EIA used to identify *M. bovis* infected lions in the Serengeti (Cleaveland et al. 2005) and the small sample size (n=6) reported on by Kirberger et al. (2006).

Subsequently Trinkel et al. (2011) considered that the proposed increase in susceptibility to *M. bovis* infection of lions with FIV (Keet et al. 1996) was indeed true for KNP lions. They compared this with the case of the Serengeti lions (Cleaveland et al. 2005) and concluded that *M. bovis* -FIV co-infection need not necessarily become an important problem in the HiP (Trinkel et al. 2011).

Additionally, *M. bovis* diagnostic tests utilising the CMI response could possibly be affected by the fact that FIVple is supposed to diminish the levels of CD4 subsets (Roelke et al. 2006; Keet et al. 2010). While looking at the viability of intradermal tuberculin testing as an ante-mortal diagnostic tool, Keet et al. (2010) concluded that FIVple co-infection did not significantly affect the intradermal skin test results.

It is clear that much more follow-up studies on lions infected with either FIV or *M. bovis* or both is needed to arrive at definitive conclusions about the possible synergies between FIV and *M. bovis.*

8. Conclusion and knowledge gaps

Relatively little has been published on *M. bovis* infection and ensuing pathology in lions. The interplay between a host and pathogenic microbe in a free-ranging ecosystem is highly complex and calls for a multidimensional and integrated approach in order to draw definitive conclusions. This review set out to critically review all publications on lion infection with *M. bovis* and tuberculosis and to identify crucial gaps in our knowledge which need to be addressed. Such knowledge gaps currently exist for the source and route of infection, and disease pathology. This includes our lack of understanding of host susceptibility, transmission directions (horizontal and vertical) and host status (maintenance, spill over or end host). We have no evidence concerning the mycobacterial dose and frequency of exposure required to establish infection and subsequent disease pathology in lions, factors which cause a lion to progress from infected to clinically diseased. We do not know whether some lions are resistant to *M. bovis* or what effect environmental stress could play in the onset and advance of active disease. While wasting and debilitation is strongly associated with end stage tuberculosis cases there are some suggestions that these are not *M. bovis* specific (Ide, 2002) end conditions. Little knowledge is available on the mechanism underlying emaciation and what effect this has on reproductive competence. At this time, there are contradictory findings and opinions on the effects of *M. bovis* on lion populations and population stability or size. We do not know with any confidence whether co-morbidities such as feline immunodeficiency virus (FIV) impact severely on individual animals or populations. Even our diagnostic ability in lions is poor and we are not confidently able to distinguish between exposure, infection and clinical disease in the lion. Such knowledge is vital to our approach to disease management of lions, and successful conservation strategies. Until such time as knowledge gaps such as those identified in this review can be addressed, management and disease control decisions will therefore largely be based on assumptions rather than robust scientific information.

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1.2 Further commentary

The aim of this review was to compile what is known of TB in lions in one document in order to aid different stakeholders to relatively easily have access to this information or references thereof. This also served the purpose of outlining areas in the field that are in need of more research that can aid in a better understanding of if/how *M. bovis* is affecting lions at individual and population level. Simultaneously, for the purpose of this thesis, it served as a general introduction to the field of lion TB research.

There were, however, some shortcomings in the review only identified after publication and as the current study progressed. Discussion of these shortcomings or reference to where they have been discussed elsewhere in the thesis follows:

- This review compiled reports of tuberculosis in both captive and free-ranging lions. The importance of looking at both these demographics to understand the disease in lions was not discussed in the review. By looking at TB cases in both captive and free-ranging lions a more complete picture can be formed of the disease in lions. Studies of human TB (due to *M. tuberculosis* or *M. bovis*) have shown that differences in demographics and environmental factors plays a role in the susceptibility of different sectors of the human population to *M. tuberculosis* or *M. bovis* infection and/or disease (Bottasso et al. 2010; Cleaveland et al. 2007; De Garine-Wichatitsky et al. 2013; de la Rua-Domenech, 2006; Frossbohm et al. 2008; Waters et al. 2014). Similarly, the varied circumstances and environmental factors

experienced by captive and free-ranging lions might either directly or indirectly affect an individual's or population's susceptibility to *M. bovis* infection or disease, and possibly also play a role in the course of disease progression. Additionally, captive lions, in general, are housed in a more controlled environment than free-ranging lions, thereby opening the possibility to better describe or aid in the understanding of observed infection or disease processes.

- The section on diagnosis of TB in lions (Section 5 in article) did not cover the use of serological TB Stat-Pak assay in lions or the development of an antigen stimulated gene expression assay. These, together with some elaboration on some limitations and advantages in the use of the different diagnostic tests will be discussed in more detail in Chapter 2.

- A further aspect of diagnosing TB in lions not included in the review was the possible limitations in obtaining viable blood samples for cytokine/chemokine based diagnostic assays. This becomes relevant for any blood- based biomarker analyses. Depending on the type of assay the blood sample is intended for, differences in obtaining, handling, and processing of the blood might be needed. For example, complicated laboratory based assays, such as the IFN-γ ELISA, would require the assay procedure to be started ideally within eight hours of sampling fresh heparinised blood but not more than 28 hours after sampling (de la Rua-Domenech et al. 2006). With free-ranging lions often not in close proximity to suitable laboratories it becomes a logistical difficulty and limitation to get viable blood samples to the laboratory. In this regard, use of or development of assays that can be conducted at the point of sampling or with initial sample processing outside of a dedicated laboratory (Miller et al. 2012; Parsons et al. 2012) might be better suited for lion TB diagnosis .

- Under section 5 on *M. bovis* diagnostics for lions, the following comment was made: "To our knowledge other clinical samples such as urine, faeces, saliva and nasal mucus has not been investigated as sources of organisms for culture." This statement could be interpreted as saying that this has not been done for any species. It should therefore be noted that this statement refers only to studies in lions.

- Under section 6 in the article a statement was made stating that "From the available literature it is clear that lions are spillover hosts but it is unclear if lions are end or maintenance hosts." In this regard it should be noted that the publication by Miller et al. (2015) showed that lions are able to shed viable bacteria and therefore supports the possibility that lions could serve as maintenance hosts.

- The importance of considering co-infection with FIV in *M. bovis* investigations was not very clear. FIV is a lentivirus that disrupts immune function in domestic cats and has also been accepted as model of HIV infection (Gómez et al. 2011). This immune compromising ability of FIV (Gómez et al. 2011) and the fact that HIV in humans is considered a risk factor for TB (Corbett et al. 2003; de la Rua-Domenech, 2006; Gupta et al. 2011) necessitates the investigation into the possible role FIV might play in lions' susceptibility to *M. bovis* infection or

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disease. Whether FIV has a negative effect on lion health (independent or in the presence of *M. bovis* infection) remains unclear. On the one hand the report by (Packer et al. 1999) suggests that the FIV strain present in lions does not negatively affect lion populations. This is speculated to be due to a longer evolutionary time frame in which lions and the virus had time to co-adapt compared to domestic cats (or humans in the case of HIV) where the viral infection is likely relatively new, in an evolutionary time scale (Carpenter & O'Brien 1995; Packer et al. 1999). Additionally, the study by Keet et al. (2010) suggested that the presence of FIV did not negatively affect the ability of the ITT as a *M. bovis* diagnostic test. On the other hand, Keet et al. (1996) cautioned that FIV infection in lions from the KNP might prove to increase the susceptibility of lions to *M. bovis*. Immune system alterations due to FIV (Bull et al. 2003; Roelke et al. 2006; Roelke et al. 2009) and pathologies similar to that described in human HIV and domestic cat FIV patients has been reported for lions (Roelke et al. 2009). In line with these findings of pathologies in FIV infected lions with similar to those seen in HIV patients, Roelke et al. (2009) cautioned that although FIV infected lions might do well under normal circumstances they may be potentially more sensitive, compared to FIV uninfected lions, to secondary assaults from other diseases such as canine distemper virus or tuberculosis.

1.3 Aims of this study

As was shown in the review, there still exist many gaps in the knowledge of tuberculosis in lions. The aim of this thesis was to initiate research into some of these knowledge gaps. Due to the overall lack of knowledge regarding underlying metabolic processes associated with *M. bovis* infection or disease in lions in conjunction with the lack of biomarker assays specifically developed for lion samples this thesis will not follow a purely hypothesis driven approach. Instead, while in some instances hypotheses might be presented, this thesis will aim to generate data that will contribute to the formation of directed hypotheses for further study. This thesis, under the main aim to initiate research into the possible effects of *M. bovis* on various lion metabolic systems, will be guided by the following additional aims or objectives:

1) Is it possible to make use of easily obtainable, commercially available assays or laboratories, not specifically designated for analyses of lion samples, to generate usable data for the biological markers of the immune/inflammatory response, energy metabolism, and reproductive endocrinology?

2) Will it be possible with the data generated to distinguish between lions of different *M. bovis* exposure or presumed infection status?

3) Will it be possible with the current study model to generate relevant knowledge to describe *M. bovis* infection or disease effects on the different lion metabolic systems studied?

4) Identify possible relationships between the different biological markers that could potentially indicate unexpected interactions, confirm usability of the assays, and/or give new insights into the possible effects of *M. bovis* in lions that could aid the direction of future research.

Importantly, the above mentioned investigation will be reliant on the accurate diagnosis of infection and/or disease. However, no single diagnostic test currently has the ability to give a comprehensive antemortem picture of the infection state or disease progress within an individual lion. The following hypothesis is presented – "The use of multiple antemortem diagnostic tests will reduce the need for necropsy in order to classify the studied lions into cohorts of different *M. bovis* disease and/or infection categories". The next chapter, and start of this investigation, will therefore be focused on the diagnosis of *M. bovis* in the current study populations with the aim of categorising the lions sampled into different cohorts from which to do a comparative investigation into the different metabolic systems.

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Chapter 2

Diagnosis of *M. bovis* infection in lions

2.1. Introduction

Mycobacterium bovis infections have been confirmed in lions (*Panthera leo*) (Cleaveland et al. 2005; Eulenberger et al. 1992; Keet et al. 1996; Michel et al. 2009; Morris et al. 1996). However, much knowledge is still needed to better understand the effects of *M. bovis* on lion populations (Viljoen et al. 2015). A clear understanding of *M. bovis* infections and subsequent tuberculosis disease (TB) is needed to aid policy makers and conservationists in implementing management plans for infected lion populations. At this point it should be noted that for the purpose of this study distinction is made between *M. bovis* infection and disease. This was done in line with the definitions of terminology adopted by the Bovine Tuberculosis Working Group when investigating different disease models for lions (Keet et al. 2009). The definitions were as follows: infected – bovine tuberculosis organisms will colonise an exposed individual but without any clinical effects or symptoms; diseased – the exposed individual becomes infected and displays clinical symptoms of the disease (Keet et al. 2009).

One of the problems encountered in the study and management of *M. bovis* in lions is the ability to accurately determine the status and progression of *M. bovis* infection and disease in living lions (Viljoen et al. 2015). Additionally, as with many other species, the progression of *M. bovis* infection to disease in lions can be slow, with sub-clinically infected animals appearing healthy (Keet et al. 2010). It seems as though clinical signs only appear in the advanced stages of disease (de Lisle et al. 2002; Keet et al. 2009; Keet et al. 1996; Keet et al. 2010; Miller et al. 2015; Morris et al. 1996). Considering the chronic nature of the disease and the lack of knowledge on the infectiousness of diseased animals, the possibility that potential transmission occurred to other individuals before observation of clinical signs exists. Additionally, some of the clinical signs usually associated with TB are not TB specific and can also be caused by other infections or ailments (Viljoen et al. 2015). Therefore, the sensitivity and specificity of diagnostic protocols are key to addressing the knowledge gaps of lion TB and making informed conservation management decisions.

To date, the gold standard diagnostic test is culture based, usually from tissue samples obtained post-mortem, and further identification by bacteriological or molecular methods. This is also the method against which other diagnostic protocols are tested (Viljoen et al. 2015). Because lions are classified as vulnerable under the IUCN Red List of Threatened Species (Nowell et al. 2012), post mortem diagnosis is not ideal for conservation management decisions. This becomes relevant when trying to conserve smaller populations, since removal of individuals for post mortem diagnosis would be detrimental to the lion population. Important data and thereby added value could be gained from having both sensitive and specific antemortem diagnostic tests.

Some attempts have been made to develop antemortem diagnostic tests for lions with various levels of success. These include the culture of bronchoalveolar lavage (BAL) samples (Miller et al. 2015), ELISA/EIA antibody tests using *M. bovis* and *M. avium* antigens (Cleaveland et al. 2005; Keet et al. 2010; Morris et al. 1996), the intradermal tuberculin skin test (ITT) (Keet et al. 2010), and serology tests by means of the TB Stat-Pak and the Dual Path Platform (DPP) VetTB assay (Miller et al. 2012). Progress has been made in cloning and sequencing lion interferon-gamma (IFN-γ) thereby laying the foundation for the possible development of lion specific IFN-γ assay (Maas et al. 2010). A recent study investigated the use of the QuantiFERON®-TB Gold (QFT) tubes to pre-treat whole blood samples by stimulating them with peptides simulating the ESAT-6, CFP-10 and TB7.7 antigens. This stimulated blood was then subjected to a gene expression assay (GEA) to measure lion specific blood based immune responses to the mycobacterial antigens (Olivier et al. 2015).

Many of the above mentioned diagnostic tests are reliant on one or more biomarkers of the immune response. Different animal species seem to show differences in the development of immune responses to mycobacterial infections. For example, elephants develop a strong humoral response whilst primates and wild boar develop an earlier cell-mediated immune response (CMI) with antibodies only produced later in the infection (Miller et al. 2012). Knowledge of the pathogenesis of *M. bovis* infection in lions is needed to interpret the results obtained from different diagnostic procedures. In this regard Miller et al. (2012) suggested that correlation of the distribution of lesions with the results of diagnostic procedures might provide some insights. They speculated that very early stages of infection may be too localised to stimulate a systemic immune response, resulting in negative test results. In most animal species, CMI precedes the humoral response and it is only as the infection progresses and pathological lesions become more advanced that a generalised immune response develops. Furthermore, some animals might have immature or compromised immune systems due to severe disease, individuals being either very old or very young, harbouring other viral or parasitic co-infections, anergy, or therapeutic interventions, that could lead to false negative diagnostic results (Miller et al. 2012).

In this study only the BAL, ITT, TB Stat-Pak, and GEA diagnostic tests were used and therefore only these will be discussed.

Development of the lion ITT was done by adapting the cattle ITT. The main alterations were the use of 0.2 ml of purified protein derivative (PPD) per injection site (double that recommended for cattle) and although both the Avian and Bovine PPD are injected at separate sites on the body, only the inflammatory response at the Bovine PPD injection site is considered. Lions that showed a 2 mm skin thickness increase at the bovine PPD injection site were considered *M. bovis* positive (Keet et al. 2010). Validation of the lion ITT was done by mycobacterial culture of samples obtained after necropsy. Keet et al. (2010) showed that the ITT was 86.5% sensitive and 81.25% specific for lions tested in the Kruger National Park (KNP). In this study they encountered seven lions that did not show a positive ITT result but were all confirmed *M. bovis* positive through culture

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methods. Additionally, six animals showed an ITT response but were not confirmed *M. bovis* positive (Keet et al. 2010). It must be noted that even if culture is regarded as a "Gold Standard", the culture result is dependent on at least two major factors, namely, that samples containing adequate numbers of viable bacilli are collected and delivered to the laboratory in good condition, and that at least some viable bacilli survive the standard decontamination procedure prior to culture. Negative culture therefore does not exclude the possibility that live bacilli and disease exists in a host. All seven of the ITT non-reactor lions were emaciated with two animals having characteristic tuberculous pulmonary lesions (Keet et al. 2010). Keet et al. (2010) suggested that this was probably akin to anergy in cattle with advanced or generalised tuberculosis and temporarily in animals experiencing stress. Of the six bacteriologically negative lions, two were emaciated with distinctive macroscopic pulmonary lesions that were histologically suggestive of mycobacteriosis. Two of the four lions that had a fair body condition were infected with non-tuberculous mycobacteria (NTM). All six of these lions originated from habitats with high and medium *M. bovis* infection pressure (Keet et al. 2010).

A disadvantage of the ITT is the inability to distinguish between different possible infection states (infected, sub-clinically infected, uninfected but previously infected, exposed to *M. bovis* but not infected). Additionally, the ITT also does not differentiate between different stages of disease (Keet et al. 2010). In order to address the problem of non reactor lions, Keet et al. (2010) suggested the use of serological diagnostic tests in concert with the ITT to increase the sensitivity.

The one step lateral-flow test (TB Stat-Pak, Chembio Diagnostic Systems, Inc., Medford, New York, USA) for rapid detection of specific antibodies has been successfully implemented to identify *M. tuberculosis* infections in non-human primates (Lyashchenko et al. 2007) and *M. tuberculosis* or *M. bovis* infections in elephants (Greenwald et al. 2009). Additionally the study by Rhodes et al. (2011) found promising results when using the TB Stat-Pak assay for the antemortem diagnosis of feline TB in domestic cats.

The TB Stat-Pak assay is a serological assay. It works on the premise that antibodies to *M. bovis* or *M. tuberculosis* in blood samples would bind to mycobacterial antigens (ESAT-6, CFP10, and MPB83) printed on the assay membrane. This binding would result in a blue indicator line appearing on the membrane in addition to the positive control line. It is easy to use, gives results within minutes and can be used in-house or in the field (Miller et al. 2012). Miller et al. (2012) used the TB Stat-Pak and the DPP assays to assess whether detection of *M. bovis* antibodies is a valid screening test for TB in lions. In their preliminary study, Miller et al. (2012) were able to retrospectively identify 64% of culture confirmed *M. bovis* positive lions with the TB Stat-Pak and 63% with the ITT. When the ITT and TB Stat-Pak results were combined, 73% of *M. bovis* culture positive animals were identified. None of the culture negative animals tested false positive with the TB Stat-Pak. Miller et al. (2012) concluded that the use of rapid antibody detection assays in the field showed promise as a screening tool for *M. bovis* infection in lions and may also be useful tools for future epidemiological studies within lion prides and populations and in TB surveillance

research. Due to the preliminary nature and small sample size (n = 14) of the study, Miller et al. (2012) did not establish specificity and sensitivity values of the TB Stat-Pak assay for lions.

If lions are indeed similar in their immune response to most other animal models with antibodies produced only at a later stage of infection/disease, then positive TB Stat-Pak results are likely to be strongly suggestive of current infection or disease. This is, however, not a given. For example, we may hypothesize that if the lion lives in an area with high infection pressure, multiple infection events might also lead to higher antibody concentrations in the blood, even though the lion is not experiencing current infection or disease.

Another blood based diagnostic assay is the QFT gene expression assay (GEA) developed by Parsons et al. (2012) for the detection of *M. bovis* infections in free-roaming African buffalo (*Syncerus caffer*). In preparation for the GEA, heparinised whole blood (WB) is incubated in the NIL and TB-antigen QuantiFERON®-TB Gold tubes. The NIL tube contains saline and the TB-antigen tube contains peptides that simulate the *Mycobacterium tuberculosis* complex (MTC) antigens ESAT-6, CFP-10, and TB7.7. Incubation of the WB with the MTC antigens elicits an immune response in the blood resulting in the stimulation of IFN-γ production. Instead of measuring the IFN-γ levels in the WB the GEA measures up-regulated IFN-γ gene transcription. Using this data the difference in IFN-γ gene expression can be calculated between the NIL and TB-antigen tubes thereby giving the GEA result. For African buffaloes the IFN-γ gene was significantly up-regulated in the TB-antigen tubes for *M. bovis* positive animals compared to *M. bovis* negative animals (Parsons et al. 2012). Olivier et al. (2015) adapted this GEA for lions. In this study, the monokine-induced by gamma interferon (MIG/*CXCL9*) gene was identified as a possible target gene in lion blood while using *YWHAZ* as a reference gene. Using this GEA Olivier et al. (2015) were able to discriminate between lions from populations experiencing different *M. bovis* and MTC exposure pressures. Additionally, different cut-off GEA values to distinguish between animals with a high probability of being *M. bovis* infected (lenient cut-off, GEA > 27), suspect of infection (a GEA response of between 5 and 27) or uninfected (stringent cut-off, GEA < 5) were proposed (Olivier et al. 2015). However, Olivier et al. (2015) mentioned that the cut-off values may change over time as more animals are tested with this method. Using these cut off values it might be possible to gain some insights into the infection status of a lion. However, drawing conclusions on disease status will be more difficult due to a lack of knowledge pertaining to what causes an individual to transfer from infected to diseased. Additionally, due to the blood being stimulated with MTC antigens, increased gene activation might be present where lions were/are infected with MTC species other than *M. bovis*. Therefore, knowledge of the geographical location and expected MTC type of exposure is also needed when interpreting the results.

All of the above mentioned antemortem diagnostic tests provide an indirect means of identifying *M. bovis* infected lions. At this time, only tracheobronchial/bronchoalveolar lavage (BAL) provides a reasonable direct method for obtaining biological material for cultural and molecular identification of viable *M. bovis* organisms in the respiratory tract of live lions (Miller et al. 2015).

Such a study was conducted in the KNP on 134 lions. One of the objectives of the study was to determine if lions could potentially be infectious. Therefore BAL samples were subjected to culture diagnostic procedures to identify animals that could be shedding viable *M. bovis* bacteria. PCR was not done on the BAL samples, although this might increase the sensitivity of identifying *M. bovis* positive animals (Miller et al. 2015). By attempting cultures of the BAL samples, Miller et al. (2015) identified 6% (8/134) of living lions as infected with *M. bovis*. The fact that most of the *M. bovis* culture positive animals were adults (median age 8.75 years) was deemed consistent with the chronic nature of mycobacterial infections and was similar to findings by Keet et al. (2000). Additionally, shedding of mycobacteria in respiratory secretions is associated with active pulmonary disease in a variety of species infected with *M. tuberculosis* or *M. bovis* (Miller et al. 2015). This led Miller et al. (2015) to suggest likely progression to active pulmonary disease of the *M. bovis* BAL culture positive lions. Of the *M. bovis* BAL culture positive lions, only one did not show clinical signs consistent with tuberculosis (Miller et al. 2015).

Using this labour intensive technique, Miller et al. (2015) managed to identify potentially infectious lions. However, the number of *M. bovis* infected animals is in all likelihood higher due to the expected intermittent shedding of viable organisms, poor recovery of organisms due to the inability to draw a sample from the entire lung, and loss of bacterial viability associated with sample handling and transportation under very difficult field conditions (Miller et al. 2015). The authors did, however, make suggestions on aspects that need to be further investigated to improve this diagnostic method.

As mentioned before, the majority of diagnostic tests rely on the immune response of the animal to facilitate diagnosis. Other diseases that are immune-compromising most likely need to be taken into account if the *M. bovis* diagnostic results are to be properly interpreted. One such disease is FIV (Feline immunodeficiency virus). Consensus about the detrimental effects of FIV on lions has not been reached. However, FIV has been shown to deplete CD4+ cell subsets (Roelke et al. 2006), cells that at least in cattle play a part in IFN-γ production (Pollock et al. 2001) while in human TB, CD4+ has immune system memory functions (Adekambi et al. 2012). Depletion of this cell subset could possibly have an effect on diagnostic tests that rely on the responses of these cells. The exact role of the CD4+ subset in lion immune function is unknown. Keet et al. (2010) found that FIV did not affect the ITT. FIV status was not considered in the TB Stat-Pak (Miller et al. 2012) or the GEA (Olivier et al. 2015) studies.

The main aim of this thesis is to gain insights into the possible effect of *M. bovis* infection and/or disease on lion immune/inflammatory responses (Chapter 3), energy metabolism (Chapter 4), and reproductive endocrinology (Chapter 5). The basis of this interrogation is reliant on the accurate diagnosis of infection and/or disease. However, no single diagnostic test currently has the ability to give a comprehensive antemortem picture of the infection state or disease progress of an individual live lion. Therefore, an important aim for this part of the study will be to categorise the

lions sampled into different cohorts from which to compare the above mentioned markers. In order to do this, the diagnostic tests will be analysed and discussed independently and co-dependently.

2.2. Materials and methods

2.2.1 General introduction and sourcing of sample animals

The initial design of the current study was to serve as an add-on study aimed at adding value to a lion demographic study undertaken by SANParks (South African National Parks) in the Kruger National Park (KNP), South Africa. This study was originally conceived to be a cohort design, where a collaboration of researchers focussed on approximately 22 lion prides that would be captured, tested for a variety of diseases and where each animal would be held for at least 72 hours for various tests, observations and diagnostics tests. There would have been follow up work done months later on the same animals to re-evaluate lion health and numbers. This thesis work began based on the detailed and extensive planning of such a "lion survey". Unfortunately, this comprehensive lion survey was not completed, since rhinoceros poaching in the KNP escalated suddenly, making the cost, logistics, and personnel availability and safety for an extensive lion study impossible. The work in this thesis therefore has limitations of low numbers of animals and controls that had to be sampled from a number of locations other than and including the KNP.

To facilitate a comparative study, lions were sampled from distinct populations separated geographically and presumed to be under different *M. bovis* exposure pressures. Lions were sourced from the Kruger National Park (KNP), two facilities of the National Zoological Gardens (NZG), viz the public zoo in Pretoria and Rietvlei Nature Reserve, the Ukutula Lion Centre (Ukutula), and two privately owned lions (PVT). The lions sampled were divided into two main categories, namely wild caught (KNP) or captive populations (NZG, Ukutula, and PVT). The southern part of the KNP has a high prevalence of *M. bovis* in the African buffalo population, an important prey species for lions, especially during the dry season (Ferreira & Funston, 2010). All the KNP lions originated from the southern part of the KNP (South of the Sabi River). The wild/KNP lions could therefore also be classified as originating from an area with high *M. bovis* prevalence or experiencing a presumed high infection pressure (likely to be *M. bovis* exposed). The captive lions were housed in enclosures with no direct contact with humans, or other live animals and animal products except for when they were fed. Feeding of the captive lions was regulated, such that they received a diet of whole chickens and sometimes carcasses of donkeys, horses, cattle or other ungulates. Efforts were made to ensure that the captive lions were not fed carcasses with transmittable diseases. Given that the prevalence of *M. bovis* in domestic stock in South Africa is reported to be extremely low[2], the captive lions could therefore be considered to be unexposed. However, the Pretoria zoo facility of the NZG where lions were sampled have on previous

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[2] A total of 256 bovine TB cases were reported in South African livestock from the beginning of 2010 to the end of 2014. Considering an estimated 14 million head of cattle in SA, this is a very small proportion of the overall South African domestic livestock population (Personal communication, Dr A Cloete, Department of Agriculture, Forestry and Fisheries, South Africa).

occasions had sporadic cases of other animal species infected with *Mycobacterium tuberculosis* complex (MTC) species, but not *M. bovis* (Michel et al. 2003). The level of diversity of the mycobacterial species suggested that infection was probably due to exposure to, and a close proximity to the public (Michel et al. 2003). The NZG and the Ukutula lions sampled in this study experienced similar situations of exposure to the public. Two of the NZG lions and all of the Ukutula lions were hand reared. The likelihood therefore exist that the Ukutula and NZG lions are sporadically exposed to MTC species other than *M. bovis.*

It should be noted that the unexpected necessity to make use of captive lions in order to facilitate a comparative study unfortunately introduced the likelihood that some bias might be present due to differences in management, housing, and feeding of the lion populations at the different geographical locations.

Due to the different management strategies at the various locations, different methods and procedures were employed to obtain the relevant samples (See sections 2.2.2, 2.2.3, and 2.2.4 for the specifics of the sampling procedures at each locality). Ethical clearance and permission for sampling were obtained from all participating parties (Stellenbosch University Research Ethics Committee, SU_ACUM12_00026; South African National Parks, VHEPD 1113; National Zoological Gardens of South Africa, Pretoria, NZG/ P12/12; University of Pretoria Animal Use and Care Committee, EC071-12). The Ukutula lions were under private ownership and consent to do the research was obtained from the owner. In all the cases and for all the localities, qualified veterinarians were in charge of administering the necessary drugs and looking after the welfare of the animals.

2.2.2 Kruger National Park sampling

This project connected in with several other projects that added on to a lion demographic study that was conducted in the KNP. Sampling took place during the Southern hemisphere winter months of 2013 (June and August). As part of the demographic study, at least one dominant female in each pride had a radio telemetry collar fitted and it was therefore possible to track the different prides. When a pride was selected, a baited call up station was set up with call ups commencing just after sunset. Lions attracted to the bait station were darted with anesthetising drugs and once adequately immobilised they were loaded on vehicles and transported to a processing station situated close by. Anesthetising drugs were made up of a combination of Medetomidine and Zoletil. Juvenile lions (< 4 years) received 3 to 5 mg Medetomidine in combination with 60 to 150 mg Zoletil. Adult lions (≥ 4 years) received 3 to 6 mg Medetomidine in combination with 100 to 200 mg Zoletil. Note: Since the mass of the lions targeted were unknown the veterinarian conducting the procedure used his/her own discretion as to the dose needed to sedate each animal. Different veterinarians were responsible on different occasions, each with their own preferred mixing ratio for the anesthetising drug cocktail.

On arrival at the processing station, blood samples for diagnostic assays and biomarker analyses were taken. This included samples for TB diagnostics (discussed later in this chapter) as well as for analyses of biological markers (discussed in relevant chapters). After blood samples were taken a thorough body condition assessment was done for each lion. Bronchoalveolar lavage (BAL) was also performed on all lions (Miller et al. 2015). The lions were weighed and body measurements were taken. Unless already done, lions were branded and received a microchip for future identification. Photographs were also taken of the face and brandings to serve as reference for future identification. Finally a tuberculosis intradermal skin test (ITT) was administered to the lions.

The processed lions were then either placed in temporary holding facilities or the adult lions fitted with radio telemetry collars to facilitate recapture of the lions after 72 hours for the reading of the skin test.

After 72 hours the lions were again anesthetised and the skin test results read by measuring the skin fold thickness at the shaven areas. Interpretation of ITT results will be discussed under section 2.2.5

Note: The two privately owned (PVT) lions were sampled by KNP veterinarians while assisting the owner with the translocation of the animals on a game farm close to the KNP.

2.2.3 National Zoological Gardens

Lions sourced from the National Zoological Gardens (NZG) were sampled to serve as a putative TB negative control population. Additionally these lions were also incorporated into experiments interrogating the reproductive endocrinology of lions.

Lions identified for sampling were separated from the rest of the pride and individually kept in a smaller management enclosure. Due to the nature of the experiment and available infrastructure we only worked with one lion at a time. The lion was immobilized with a combination of Medetomidine, Midazolam, and Butorphanol via remote injection dart (Daninject). The drug combination for juvenile lions (<4 years) consisted of 3 to 4 mg Medetomidine, 10 to 15 mg Midazolam, and 15 to 20 mg Butorphanol. Adult lions (≥ 4 years) received a combination of drugs consisting of 5 to 6 mg Medetomidine, 15 to 20 mg Midazolam, and 20 to 30 mg Butorphanol. Once immobilized, the lion was weighed, intubated and maintained on isoflurane in oxygen. Intravenous fluid (Ringer's Lactate) was administered at a rate of 5 to 10 ml/kg/hr. Cardiorespiratory parameters were monitored by means of ECG, pulse oximetery, capnography, indirect blood pressure and rectal temperature using a multi functional veterinary monitor (Cardell 9500 HD, Midmark Corporation, United Kingdom). The animals were maintained in a light but safe plane of anaesthesia.

A 14 gauge intravenous catheter was placed in one of the medial saphenous veins for the intermittent collection of samples for the reproductive endocrinology experiment. The catheter was flushed with heparin in saline after each sample collection.

All blood samples for TB diagnostics, energy metabolism, and immune/inflammatory system investigations were taken before the onset of the reproductive endocrinology experiment to avoid any possible effects the experiment might have on the samples. For the detailed procedure of the experiment see Chapter 5 section 5.2.2.

After completion of the experiment the BAL sample was collected (See section 2.2.5) and finally an ITT was administered on the lion before being placed back in the management enclosure and woken up. After 72 hours the lions were sedated again in order to measure the intradermal skin test. Results were interpreted as described in section 2.2.5.

2.2.4 Ukutula

Lions sourced from the Ukutula lion centre were also sampled to serve as a TB negative control population. Additionally, these lions were also incorporated into the reproductive endocrinology interrogation experiment.

Procedures at Ukutula were similar to those at the NZG. However, due to the infrastructure available, the lions were not intubated for the duration of the procedure but kept under a surgical plane of anaesthesia by administrating additional doses of anaesthetic drugs as needed. Drugs used to immobilise the lions were in the majority of cases a combination of Medetomidine and Ketamine at dosages of 6 to 12 mg Medetomidine and 150 to 200 mg Ketamine for juvenile lions (< 4 years), and 4 to 12 mg Medetomidine together with 50 to 200 mg Ketamine for adult lions (≥ 4 years). Three lions received a combination of 5 mg Medetomidine and 150 mg Zoletil. The welfare of the lions was constantly monitored by a qualified veterinarian and all work halted if the veterinarian deemed it necessary.

2.2.5 Diagnostic tests

A fundamental part of doing a comparative study is the correct diagnosis and differentiation between such healthy and diseased animals. The different diagnostic protocols utilised in this study were the ITT, BAL, QFT based GEA, and TB STAT-Pak. Additionally FIV status was also determined. The following are some details on the handling of samples and processing of the different diagnostic tests.

ITT: For both wild and captive lions, injection of PPD and measurements of the skin reactions were done by experienced veterinarians. Injection of PPD was done after all the other samples were taken and just before the anaesthesia was reversed. This was done to limit any unnecessary handling and possible interference with the ITT injection sites.

The ITT was performed by shaving a small (2 cm x 2 cm) patch of fur on each side of the neck. The skin fold thickness of each side was measured with callipers after which 0.2 ml of bovine tuberculin was injected intradermally into the shaved skin on the right and 0.2 ml of avian tuberculin injected similarly into the skin on the left side. Administration of PPDs for the captive (NZG and Ukutula) lions were 0.2 ml Avian tuberculin PPD strain D4ER (2500 IU/ 0.1 ml), and 0.2 ml Bovine tuberculin PPD strain AN5 (3000 IU/ 0.1 ml).

Interpretation of the ITT results was based on the single intradermal cervical tests described by Keet et al. (2010). With this method, the inflammatory reaction is measured at both the avian and bovine tuberculin injection site but only considering the measurement at the bovine tuberculin injection site. Care was taken not to touch or palpate the shaven areas before measurement and repeat measurements were not done as this could result in false measurements. A lion was considered *M. bovis* positive if the difference between the initial and repeat measurement at the bovine tuberculin PPD site was greater or equal to 2 mm.

BAL: All KNP lions were sampled and handled in accordance to the protocol described in Miller et al. (Miller et al. 2015). The BAL sampling of the NZG and Ukutula lions was done by qualified veterinarians and assisted by myself. A foal feeding tube was passed through the intubation tube and positioned so that the tip was at or near the bronchial bifurcation. 50 ml of warm (about 37.5 ^{0}C) sterile saline was flushed through the tube and into the mainstem bronchi. A 50 ml syringe was attached to the external end of the tube and negative pressure was applied to the syringe, while repositioning the tube in order to aspirate as much of the saline as possible. The tube was then withdrawn and the contents of the syringe and tube flushed into a sterile 50 ml conical tube. These samples were then centrifuged at 3000 rpm for 10 min and the pellet stored and frozen at -80 ºC. The BAL samples were subjected to mycobacterial culture (incubation in BACTEC^{TM} MGIT 960 Mycobacterial Detection System, Becton Dickinson; and Ziehl-Neelsen staining) and speciation protocols (Alexander et al. 2010; Miller et al. 2015) at the Division Molecular Biology and Human Genetics, University of Stellenbosch Faculty of Health Sciences.

QFT-GEA: During the sampling procedures described in sections 2.2.2, 2.2.3, and 2.2.4 approximately six ml of blood was collected into a heparinised (green top) vacutainer tube. In the KNP these samples were stored at ambient temperature until further processing later the same evening. Since sampling of the captive lions occurred during the day time, these samples were kept cool, but not chilled or on ice until further processing later the same day.

The gene expression assay (GEA) diagnostic procedure described in Olivier et al. (2015), requires specific pre-treatment of the blood samples. On arrival at the lab, the heparin blood tubes were gently inverted to re-suspend any separation of the blood that might have occurred. One millilitre blood was transferred to the QFT-NIL and QFT-TB tubes (QuantiFERON®-TB Gold tubes) using a sterile needle and syringe. The QFT tubes were gently inverted 10 times so that the blood completely covered the inner walls of the tubes. The QFT tubes with blood samples were then placed in an incubator at 37ºC for 20 hours standing upright. After incubation the whole blood sample was transferred to 2 ml sample tubes and centrifuged at 3000 x g for 10 minutes. The plasma was then carefully separated from the cell pellet and placed into separate sample tubes and frozen at – 20 ºC. Approximately 1.6 ml of RNALater® (Ambion, Austin, TX, USA) was then added to the cell pellet and gently mixed by repeated pipetting. Both the cell sample with RNALater® and the plasma sample were then placed into a -20 ºC freezer and stored until further processing. The pre-treated samples were analysed in the Division Molecular Biology and Human

Genetics, University of Stellenbosch Faculty of Health Sciences and the GEA was done in accordance with the methods described by Olivier et al. (2015).

For the TB Stat-Pak and FIV analyses blood was collected in a serum (red-top) tube and kept on ice until further processing the same day. Blood was allowed to coagulate and then centrifuged at 3000 rpm for 10 minutes. The serum was then divided into approximately 500 µl aliquots and stored at -20 ºC.

TB Stat-Pak: Serum samples, fresh or stored at -20 ºC, were used for TB Stat-pak (Chembio Diagnostic Systems, Inc., Medford, New York, USA) analyses. All samples were assayed in accordance with the manufacturer's guidelines.

FIV: As part of the larger lion study in the KNP, FIV status was determined by antibody based ELISA at the Department of Veterinary Tropical Diseases' Serology Laboratory, Onderstepoort, Pretoria, South Africa. KNP FIV results kindly supplied by Dr. D. Govender, Dr. A. Jolles, and H. Broughton (Scientific Services Ecology Division, South African National Park Services, Skukuza, Mpumalanga, South Africa, and Department of Integrative Biology, Oregon State University, Corvallis, Oregon, USA). Stored serum samples from the NZG and Ukutula lions were assayed by myself for FIV using the FeLV/FIV IDEXX SNAP Combo Plus® Test kits (IDEXX Laboratories, Maine, USA) in accordance with the manufacturer's guidelines.

2.2.6 Statistical analysis

Some of the diagnostic tests (ITT and QFT-GEA) generated quantitative data that were statistically inspected. Different groupings were compared with either a two-tailed unpaired t-test with Welch's correction (Welch test) when variances differed significantly or with a two-tailed unpaired Mann Whitney test (MW test) when variances were not significantly different. Significance of differences for categorical variables between two cohorts were analysed using contingency tables and the Fisher's exact test. When more than two categories were present contingency table analyses was done with the Chi-square test. Data were visualised and statistically analysed with the GraphPad Prism Version 4 for Windows (GraphPad Software, Inc., CA, USA).

2.3 Results

In total, 68 lions were sampled comprising 39 KNP/wild/*M. bovis* exposed lions and 29 captive/*M. bovis* unexposed lions. The captive lions were sampled from the NZG (n=13), Ukutula (n= 14), and an additional two privately owned lions. Unfortunately all diagnostic tests could not be done on some of the lions. Reasons for this include: missing ITT results due to unsuccessful recapture attempts; problems encountered with the processing (clotting) of blood or analysis (low initial RNA yield) of some of the blood samples for the GEA analyses; lack of necessary equipment prevented BAL sampling of the two PVT lions; One Ukutula animal was sampled opportunistically enabling only the GEA diagnostic test to be performed. The demographic information and diagnostic results are presented in Table 2.5.

For both the ITT and the GEA, quantitative data was generated. Therefore, the first part of the results section will be focussed on independently investigating the numerical data generated by these two diagnostic tests. This will be followed by an investigation of the diagnostic classifications the lions received using the diagnostic tests described for the current study.

2.3.1 ITT diagnostic measurements

As part of the normal ITT procedure the thickness of the skin fold at the injection site was measured before and 72 hours after PPD injection. Using this data the inflammatory increase in skin fold thickness (in mm) was calculated for both the Bovine and Avian PPD injection sites. A summary of the data can be seen in Table 2.1. As mentioned in section 2.2.5, lions that showed an inflammatory increase in skin thickness of ≥ 2 mm at the Bovine PPD injection site were considered to be ITT positive whilst lions with a lesser inflammatory response were considered ITT negative. Although only the inflammatory increase at the Bovine PPD injection site was considered for diagnostic purposes, the quantitative data generated at both PPD injection sites were inspected since this could give additional insights into the overall diagnosis and cohort classifications used in the current study. The most severe inflammatory increase was observed at the Bovine PPD injection site in the KNP/wild ITT positive lions. At the Avian PPD injection site, the most severe inflammatory increase was seen in ITT positive Ukutula lions. Comparisons of the inflammatory response at the Bovine PPD injection site with that of the Avian PPD injection site showed a significantly greater response at the Bovine PPD site in only the KNP/*M. bovis* exposed ITT positive lions (Paired t-test, $p < 0.0001$). One way ANOVA's showed that the inflammatory response at the Bovine PPD site for ITT positive lions differed significantly between the different lion populations ($p < 0.0001$). Although the inflammatory response at the Bovine PPD site in ITT negative lions did not differ significantly between KNP and NZG or Ukutula lions it was significantly greater in NZG lions compared to Ukutula lions (MW test, $p = 0.0485$).

At the Avian PPD injection site significant differences in inflammatory responses were observed between ITT positive and ITT negative animals for both wild and captive lions. The inflammatory response for both ITT positive and negative lions at the Avian PPD injection site was significantly greater in captive/*M. bovis* unexposed lions than in wild/*M. bovis* exposed lions.

Linear regressions were done with the data obtained from ITT positive lions, by plotting the inflammatory response at the Avian PPD injection site against the response at the Bovine PPD site (Figure 2.1). The slopes of the regression lines differed significantly ($p = 0.0069$) between the *M. bovis* exposed and unexposed lions. A stronger correlation was observed between the responses at the Bovine and Avian PPD injection sites of unexposed lions (Pearson, $r^2 = 0.5164$) than for exposed lions (Pearson, $r^2 = 0.3797$).

Table 2.1: Summary of the mean inflammatory response (in mm) at the ITT injection sites according to location and suspected *M. bovis* exposure. Values given as (n) Mean ± SD.

	Bovine PPD		Avian PPD	
	[1]ITT +	ITT -	ITT +	ITT -
KNP/Wild/*M. bovis* exposed	(28) 6.01 ± 2.3	(8) 0.79 ± 0.87	(28) 2.14 ± 1.27	(8) 0.35 ± 0.54
Captive/*M. bovis* unexposed	(14) 2.81 ± 0.76	(14) 0.67 ± 0.71	(14) 3.39 ± 1.54	(12) 1.58 ± 0.59
NZG	(5) 2.86 ± 0.78	(8) 0.94 ± 0.58	(5) 3.18 ± 0.95	(8) 1.74 ± 0.61
Ukutula	(9) 2.78 ± 0.79	(4) -0.03 ± 0.71	(9) 3.81 ± 1.69	(4) 1.25 ± 0.44

[1] ITT *M. bovis* positive (+); ITT *M. bovis* negative (-)
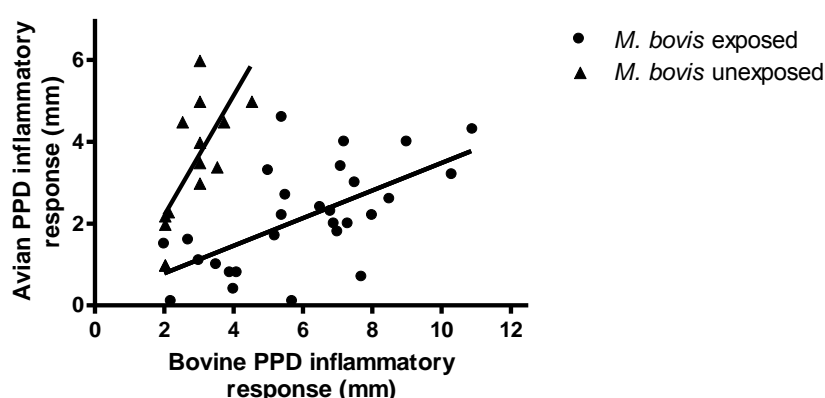
Figure 2.1: Linear regression plots of the inflammatory responses of the Avian PPD site plotted against the Bovine PPD site for ITT positive lions. Data were grouped by suspected *M. bovis* exposure.

2.3.2 QFT GEA results

Quantitative data were obtained from the GEA. Results were first compared between the different animal locations and then according to area of suspected *M. bovis* exposure. The GEA response of the KNP animals (Figure 2.2, A) had a significantly higher mean value than responses from the NZG (Welch test, p = 0.0021) and Ukutula (Welch test, p = 0.0165) lions. Animals originating from the *M. bovis* exposed area had significantly greater (Welch test, p = 0.0049) GEA responses than animals from the *M. bovis* unexposed area (Figure 2.2, B).
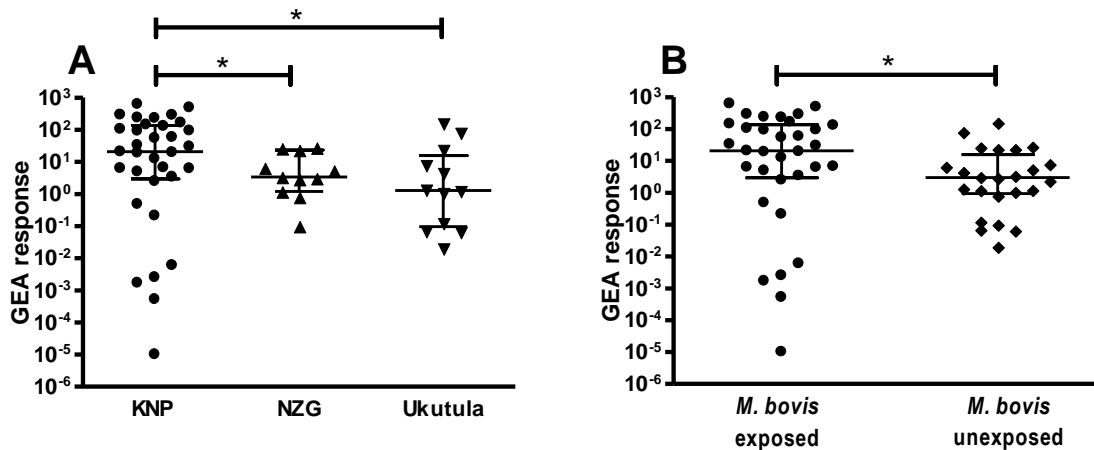
Figure 2.2: Scatter dot plots of the GEA responses (arbitrary units) for the KNP, NZG, and Ukutula lions (A) and the different suspected *M. bovis* exposure groups (B). Lines indicate the median and interquartile ranges. A - The GEA responses in KNP lions differed significantly (Welch test, *p < 0.05) from the GEA responses in respectively the NZG and Ukutula lions. B - The GEA responses of *M. bovis* exposed lions differed significantly (Welch test, *p < 0.05) from GEA responses in unexposed lions.

2.3.3 Antemortem *M. bovis* diagnostic results

Table 2.2 gives a summary of the diagnostic assay results for the lions tested in the current study. More lions (n = 42/64) were identified as being potentially *M. bovis* positive with the ITT than with any other diagnostic test. Using the cut off values suggested by Olivier et al. (2015), 13 of 57 lions were identified by means of the GEA as suspect *M. bovis* infected (assay result between 4.4 and 27) and 19 of 57 lions as having a high probability of being *M. bovis* infected (assay result > 27). When the lions were grouped according to their different places of origin a greater proportion of the KNP/wild lions tested *M. bovis* positive with all of the diagnostic tests (Table 2.2) except for the TB Stat-Pak where the same proportion (7.7 %) of NZG lions tested positive. In the case of the captive lions, a greater proportion of Ukutula lions (n = 9/13, 69.2 %) tested positive using the ITT while a greater proportion of NZG lions tested positive using the TB Stat-Pak (n = 1/13, 7.7 %). A greater proportion of NZG lions compared to Ukutula lions were classified as suspect of infection or probably infected by means of the QFT GEA. No Ukutula animals tested positive using the TB Stat-Pak.

The Ziehl-Neelsen (ZN) stain identifies only if the bacterial culture from the BAL sample are acid-fast or not. Overall only four BAL samples were ZN positive. Further speciation showed that one KNP lion BAL sample was identified as *M. bovis* positive. The other two ZN positive BAL samples from the KNP were identified as one non-mycobacterial species and one *M. intracellulare*. The ZN positive BAL sample from Ukutula was identified as *M. terrae.* Both *M. terrae* and *M. intracellulare* are considered to be non-tuberculous mycobacterium (NTM).

All of the wild/KNP lions (n = 39/39, 100 %) were FIV positive whilst only a small percentage of the captive lions tested FIV positive (n = 5/28, 17.9 %). For the captive lions, a

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higher proportion of NZG lions (n = 3/13, 23.1 %) were FIV positive when compared to Ukutula lions (n = 2/13, 15.4 %).

Table 2.2: Summary of diagnostic test results for the current study according to origin of samples. Numbers in brackets represents proportions (%) of result for animals tested per location (n)

Diagnostic test	All[1]	KNP/Wild	Captive[2]	NZG	Ukutula
ITT (n)	64	36	28	13	13
Positive	42 (65.6)	28 (77.8)	14 (50)	5 (38.5)	9 (69.2)
Negative	22 (34.4)	8 (22.2)	14 (50)	8 (61.5)	4 (30.8)
Stat-Pak (n)	67	39	28	13	13
Positive	4 (6.0)	3 (7.7)	1 (3.6)	1 (7.7)	0 (0)
Negative	63 (94.0)	36 (92.3)	27 (96.4)	12 (92.3)	13 (100)
QFT GEA (n)	57	33	24	11	12
Probable	19 (33.3)	16 (48.5)	3 (12.5)	1 (9.1)	2 (16.7)
Suspect	13 (22.8)	7 (21.2)	6 (25)	4 (36.4)	2 (16.7)
Unlikely	25 (43.9)	10 (30.3)	15 (62.5)	6 (54.5)	8 (66.7)
BAL (n)	65	39	26	13	13
ZN positive	4 (6.2)	3 (7.7)	1 (3.8)	0 (0)	1 (7.7)
ZN negative	61 (93.8)	36 (92.3)	25 (96.2)	13 (100)	12 (92.3)
FIV (n)	67	39	28	13	13
Positive	44 (65.7)	39 (100)	5 (17.9)	3 (23.1)	2 (15.4)
Negative	23 (34.3)	0 (0)	23 (82.1)	10 (76.9)	11 (84.6)

[1]All = wild + captive lions

[2]Captive = NZG + Ukutula + PVT (Since PVT consisted of only two lions a separate column was not included for them in this table)

Some gender based differences were observed. A greater proportion of females (n = 28/39, 71.8 %) compared to males (n = 14/25, 56 %) were identified as *M. bovis* positive with the ITT. All the lions that tested positive using the TB Stat-Pak were female (n = 4/41, 9.8 %) while no males (n = 0/26) tested positive. Using the GEA cut off values suggested by Olivier et al. (2015) a higher percentage of females (n = 20/34, 58.8 %) were suspect or probably infected compared to males (n = 12/23, 52.2 %). A greater proportion of females (n = 29/41, 70.7 %) were identified as FIV positive compared to males (n = 15/26, 57.7 %). Despite the greater proportions of females than males that had *M. bovis* positive diagnostic results, the observed differences were not significant (p > 0,05, Fisher's exact test on Contingency tables). The ZN positive BAL culture results were obtained from one Ukutula male and three KNP females. The *M. bovis* positive culture came from a KNP female.

Considering the diagnostic data for only the KNP lions, more females (n = 19/23, 82.6 %) compared to males (n = 9/13, 69.2 %) were identified as *M. bovis* positive by the ITT. For both male and female KNP lions, the GEA identified more animals as probably infected (n = 16/33) compared to lions suspected of infection (n = 7/33). The proportion of females (n = 11/20, 55 %) with a high probability of infection was greater than for males (n = 5/13, 38.55 %). Similar percentages of males (n = 3/13, 23.1 %) and females (n = 4/20, 20 %) were suspected of infection, although males may have a slightly higher risk. None of the above-mentioned differences for the wild lions were significant (p > 0.05) using contingency table Fisher's exact tests.

A similar situation was seen for captive lions with a greater proportion of females compared to males testing FIV positive, ITT positive, and by means of the GEA as probably infected. A greater proportion of captive males than females were classified as suspect of infection by means of the GEA. These differences were also not significant (p > 0.05) using contingency table Fisher's exact tests.

 Lions were grouped into three different age classes, namely juvenile (< 2 years), sub-adult (2-4 years), and adult (> 4 years). Similar percentages of juveniles, sub-adults, and adults were *M. bovis* positive according to ITT. No sub-adults (n = 0/9) tested positive using the TB Stat-Pak while three adults (n = 3/37) and one juvenile (n = 1/21) tested positive. The GEA test suggested that a greater proportion of adults than juveniles or sub-adults had a high probability of being infected, whilst a greater proportion of juveniles had suspected infections. None of these differences between the age classes were considered significant (p > 0.05) using contingency table Chi-square test. All the ZN positive BAL cultures were from adult lions.

Some age class related differences were observed between the different sample locations. More adult (n = 16/17) and sub-adult (n = 2/2) KNP lions were ITT positive, whilst more juvenile (n = 3/4) and sub-adult (n = 5/7) captive lions were ITT positive. These age related differences in ITT diagnosis were significant (p = 0.0256, Chi-square test) for the wild/KNP lions but not for the captive lions. Positive TB Stat-Pak diagnosis was only seen for adult KNP lions (n = 3/20) and for a captive juvenile lion (n = 1/4). Age related differences for TB Stat-Pak diagnoses were significant for the captive lions (p = 0.0446, Chi-square test) but not for the wild/KNP lions. The GEA test suggested that more adult than juvenile KNP lions were probably infected. Looking at the GEA diagnosis of the different age classes independently, equal proportions of wild juvenile lions were classified as suspects for infection (n = 5/14, 35.7 %) and as probably infected (n = 5/14, 35.7 %) while a greater proportion of adults were classified as probably infected (n = 10/18, 55.6 %) than suspect for infection (n = 2/18, 11.1 %). The GEA test identified a greater proportion of captive adults (n = 2/12, 16.6 %) than juveniles (n = 0/4) as probably infected. Looking at the GEA diagnosis of the age classes independently, a greater proportion of juveniles were classified as suspect for infection (n = 2/4, 50 %) than probably infected (n = 0/4). This was similar for captive adult lions with a greater proportion being classified as suspect for infection (n = 3/12, 25 %) than probably infected (n = 2/12, 16.7 %).Exactly the same proportion (n = 1/8, 12.5 %) of captive sub-

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adult lions were classified as either suspect of infection or probably infected. Since all the KNP lions (n = 39) were FIV positive, comparisons between the age classes could not be made. Positive FIV results were obtained for captive adult lions only (n = 5/17, 29.4 %).

2.3.3.1 Multiple positive diagnoses

A total of 52 lions were tested with all four diagnostic tests used in this study (see Table 2.3). Of these, 15 lions (comprising of 10 captive and five wild) were identified as *M. bovis* positive with just one test (ITT), whilst 11 lions were classified as probably infected by means of the GEA and also tested positive using the ITT. Nine of the lions that were classified as suspected of infection using the GEA also tested positive by means of the ITT. Of these lions with a positive ITT result and a concurrent GEA classification of suspect of infection or probably infected, only three were captive.

A total of 14 lions were tested with only three of the four diagnostic tests (see Table 2.3). One of these lions was from the KNP and tested positive with the ITT and TB Stat-Pak. This lion was confirmed to be infected with *M. bovis* by means of culture diagnosis of the BAL sample. All three KNP lions that tested positive using the TB Stat-Pak also tested positive using the ITT.

FIV status was also compared with diagnostic test results (Table 2.4). All of the KNP lions were FIV positive. Together, the ITT, TB Stat-Pak, and BAL identified 71.8 % (n = 28/39) of the KNP lions as *M. bovis* positive. 59 % (n = 23/39) of the KNP lions were, by means of the GEA, classified as suspected or probably infected with *M. bovis*. For the captive lions, a greater proportion of FIV negative animals tested positive for *M. bovis* with the ITT, and TB Stat-Pak, compared to FIV positive lions. Similarly, using the GEA, a greater proportion of captive FIV negative lions were identified as probably or suspected of being infected with *M. bovis* than were FIV positive lions.

Considering the quantitative GEA data, some associations were observed between the GEA and other diagnostic test results. In general, the GEA responses of ITT positive lions were significantly greater than that of ITT negative lions (Welch test, p = 0.0279). However, within the different sample locations and *M. bovis* exposure groupings, none of the GEA responses differed significantly between ITT positive and ITT negative lions.   Comparisons of GEA responses for ITT positive lions whilst taking the animals location or suspected *M. bovis* exposure in to account, showed that the responses of KNP lions were significantly greater than respectively NZG (Welch test, p = 0.0027) and Ukutula (Welch test, p = 0.0132) lions (Figure 2.3, A). Similarly, the GEA responses of ITT positive lions from the *M. bovis* exposed area were significantly greater than that of lions from the *M. bovis* unexposed area (Welch test, p = 0.0064) (Figure 2.3, B). The GEA responses of ITT negative lions were not associated with location or suspected *M. bovis* exposure.

Table 2.3: Summary *M. bovis* diagnostic status of lions for which more than one diagnostic test was done.

Lions tested with all four diagnostic tests (n = 52)	(n)	16SrRNA species identification
Only ITT positive	15	
Only TB Stat-Pak positive	0	
Only ZN Positive	1	NTM (*M. terrae*)
Only GEA probable	4	
Only GEA suspect	3	
GEA suspect + TB Stat-Pak positive	1	
GEA probable + ITT positive	11	
GEA suspect + ITT positive	9	
GEA probable + ZN positive + ITT positive	1	Non Mycobacteria
GEA probable + TB Stat-Pak positive + ITT positive	1	
GEA probable + ZN positive + TB Stat-Pak positive + ITT positive	1	*M. intracellulare*
Lions tested with three diagnostic test (n = 14)		
Only ITT positive	3	
Only GEA probable	1	
ZN positive + TB Stat-Pak positive + ITT positive	1	*M. bovis*

Table 2.4: Summary of FIV status data in conjunction with *M. bovis* positive diagnostic results

FIV vs. *M. bovis* diagnostics	(n)	%
KNP/Wild (FIV pos = 39; FIV neg = 0)		
FIV positive + ITT, TB Stat-Pak and/or ZN positive	28	71.8
FIV negative + ITT, TB Stat-Pak and/or ZN positive	0	
FIV positive + GEA suspect or probable infected	23	59.0
FIV negative + GEA suspect or probable infected	0	
Captive (FIV pos = 5; FIV neg = 23)		
FIV positive + ITT, TB Stat-Pak and/or ZN positive	2	40.0
FIV negative + ITT, TB Stat-Pak and/or ZN positive	14	60.9
FIV positive + GEA suspect or probable infected	1	20.0
FIV negative + GEA suspect or probable infected	8	34.8

It was not possible to discriminate (MW test, $p = 0.3625$) between TB Stat-Pak positive (83.15 ± 52.26, mean ± SD) and negative (94.53 ± 152.1, mean ± SD) lions using the GEA test. However, due to the small number of TB Stat-Pak positive lions, definitive conclusions cannot be

made. Comparisons of the GEA responses were done taking the FIV status of the lions into account (Figure 2.4). FIV positive lions had significantly higher (Welch test, p = 0.0090) GEA responses than FIV negative lions (Figure 2.4, A). The small sample size of captive FIV positive lions prevented statistically sound observations. Still, the mean GEA response of KNP/wild FIV positive lions were greater than for captive FIV positive lions (Figure 2.4, B). Within the captive population, the mean GEA response of FIV positive and negative lions was similar.
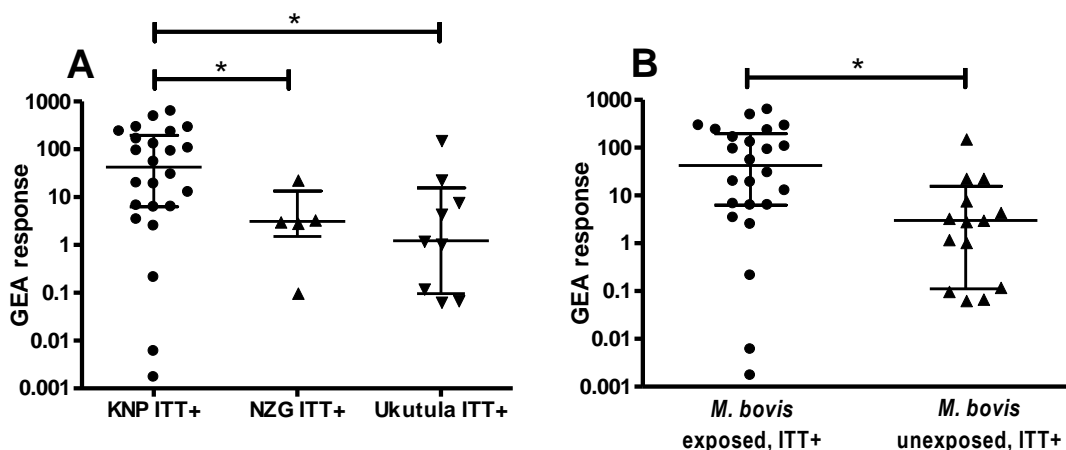
Figure 2.3: Scatter dot plots of the GEA responses (arbitrary units) for ITT positive (+) KNP, NZG, and Ukutula lions (A) and the different suspected *M. bovis* exposure groups (B). Lines indicate the median and interquartile ranges. A - The GEA responses in ITT positive KNP lions differed significantly (Welch test, *p < 0.05) from the GEA responses in respectively ITT positive NZG and Ukutula lions. B - The GEA responses of ITT positive *M. bovis* exposed lions differed significantly (Welch test, *p < 0.05) from GEA responses in ITT positive unexposed lions.
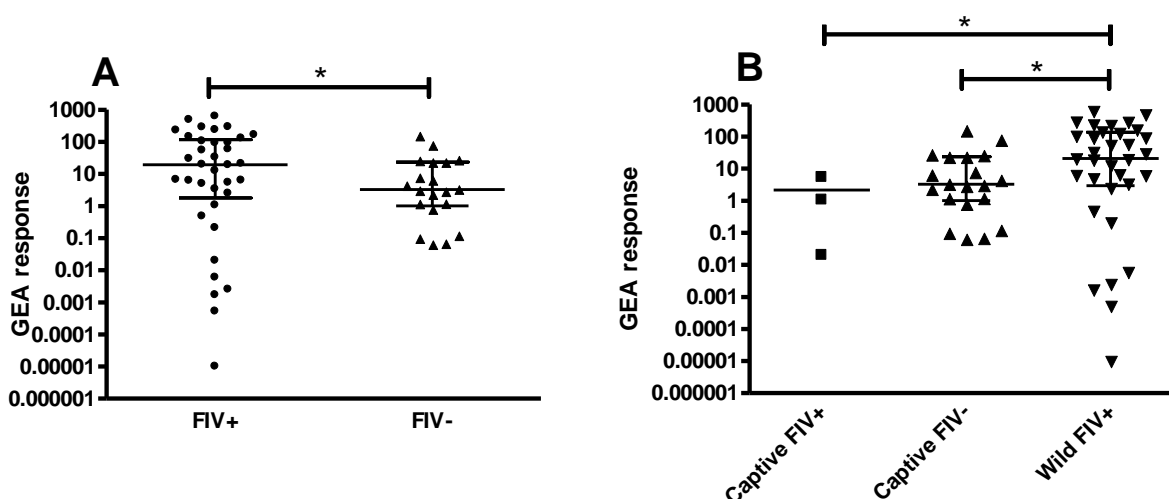
Figure 2.4: Scatter dot plots of the GEA responses (arbitrary units) grouped by FIV status (A) and according to origin and FIV status (B). Lines indicate the median and interquartile ranges. A – The GEA responses in FIV positive (+) lions were significantly greater (Welch test, *p < 0.05) than the GEA responses in FIV negative (-) lions. B – The GEA responses of wild FIV positive lions were significantly greater (Welch test, *p < 0.05) than the GEA responses in respectively FIV positive and FIV negative captive lions.

Table 2.5: Diagnostic results and demographic information of each lion sampled in the current study. (ITT = intradermal tuberculin skin test, BAL = bronchoalveolar lavage, QFT GEA = QuantiFERON®-TB Gold gene expression assay, FIV = feline immunodeficiency virus, M = male, F = female, AD = adult, SA = sub-adult, JU = Juvenile, NTM = non-tuberculous mycobacteria)

Lion #	Origin	Pride	*M. bovis* exposure	Sex	KNP Age class	ITT	TB Stat-Pak	BAL	QFT GEA classification	FIV
KNP01	Wild	Croc bridge	Likely exposed	F	AD	Positive	Negative	Negative	probable	Positive
KNP02	Wild	Croc bridge	Likely exposed	F	AD	Positive	Negative	Negative	probable	Positive
KNP03	Wild	Croc bridge	Likely exposed	M	SA	Positive	Negative	Negative	probable	Positive
KNP04	Wild	Croc bridge	Likely exposed	F	SA	Positive	Negative	Negative		Positive
KNP05	Wild	Croc bridge	Likely exposed	F	AD	Positive	Positive	*M. bovis*		Positive
KNP06	Wild	Croc bridge	Likely exposed	F	JU	Positive	Negative	Negative	suspect	Positive
KNP07	Wild	Croc bridge	Likely exposed	M	JU	Positive	Negative	Negative	suspect	Positive
KNP08	Wild	Croc bridge	Likely exposed	F	JU	Negative	Negative	Negative		Positive
KNP09	Wild	Croc bridge	Likely exposed	M	JU	Negative	Negative	Negative	probable	Positive
KNP10	Wild	Nwawitshaka	Likely exposed	F	AD	Positive	Negative	Negative	suspect	Positive
KNP11	Wild	Nwawitshaka	Likely exposed	F	AD	Positive	Negative	Negative	probable	Positive
KNP12	Wild	Nwawitshaka	Likely exposed	F	AD	Positive	Negative	Negative	unlikely	Positive
KNP13	Wild	Nwawitshaka	Likely exposed	F	JU	Negative	Negative	Negative	unlikely	Positive
KNP14	Wild	Nwawitshaka	Likely exposed	F	JU	Negative	Negative	Negative	probable	Positive
KNP15	Wild	Nwawitshaka	Likely exposed	M	JU	Negative	Negative	Negative	unlikely	Positive
KNP16	Wild	Nwawitshaka	Likely exposed	F	AD	Positive	Negative	Negative		Positive
KNP17	Wild	Nwawitshaka	Likely exposed	F	AD	Positive	Negative	Negative	unlikely	Positive

Lion #	Origin	Pride	*M. bovis* exposure	Sex	KNP Age class	ITT	TB Stat-Pak	BAL	QFT GEA classification	FIV
KNP18	Wild	Nwawitshaka	Likely exposed	F	JU	Negative	Negative	Negative		Positive
KNP19	Wild	Lower sabie	Likely exposed	F	AD	Positive	Negative	Non Mycobacterial	probable	Positive
KNP20	Wild	Lower sabie	Likely exposed	F	AD		Negative	Negative	unlikely	Positive
KNP21	Wild	Lower sabie	Likely exposed	F	AD	Positive	Negative	Negative	unlikely	Positive
KNP22	Wild	Lower sabie	Likely exposed	M	JU	Positive	Negative	Negative	unlikely	Positive
KNP23	Wild	Lower sabie	Likely exposed	F	JU	Positive	Negative	Negative	probable	Positive
KNP24	Wild	Lower sabie	Likely exposed	M	JU	Positive	Negative	Negative		Positive
KNP25	Wild	Lower sabie	Likely exposed	M	AD	Negative	Negative	Negative	unlikely	Positive
KNP26	Wild	Lower sabie	Likely exposed	M	JU	Negative	Negative	Negative	suspect	Positive
KNP27	Wild	Lower sabie	Likely exposed	M	JU	Positive	Negative	Negative	probable	Positive
KNP28	Wild	Lower sabie	Likely exposed	F	AD	Positive	Negative	Negative	probable	Positive
KNP29	Wild	Hlongo, Lukimbi	Likely exposed	M	AD	Positive	Negative	Negative	probable	Positive
KNP30	Wild	Hlongo, Lukimbi	Likely exposed	F	AD		Negative	Negative	probable	Positive
KNP31	Wild	Hlongo, Lukimbi	Likely exposed	M	AD		Negative	Negative	unlikely	Positive
KNP32	Wild	Stolznek	Likely exposed	F	AD	Positive	Negative	Negative	probable	Positive
KNP33	Wild	Stolznek	Likely exposed	F	AD	Positive	Positive	*M. intracellulare*	probable	Positive

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Lion #	Origin	Pride	*M. bovis* exposure	Sex	KNP Age class	Skin-test	STAT-pak	BAL	QFT GEA classification	FIV
KNP34	Wild	Stolznek	Likely exposed	F	AD	Positive	Positive	Negative	probable	Positive
KNP35	Wild	Stolznek	Likely exposed	F	JU	Positive	Negative	Negative	suspect	Positive
KNP36	Wild	Stolznek	Likely exposed	M	JU	Positive	Negative	Negative	unlikely	Positive
KNP37	Wild	Stolznek	Likely exposed	M	JU	Positive	Negative	Negative	suspect	Positive
KNP38	Wild	Stolznek	Likely exposed	M	JU	Positive	Negative	Negative	probable	Positive
KNP39	Wild	Stolznek	Likely exposed	F	AD	Positive	Negative	Negative	suspect	Positive
NZG 01	Captive	PTA Zoo	Unexposed	F	AD	Negative	Negative	Negative	suspect	Positive
NZG 02	Captive	PTA Zoo	Unexposed	F	AD	Negative	Negative	Negative		Positive
NZG 03	Captive	PTA Zoo	Unexposed	F	AD	Negative	Negative	Negative		Positive
NZG 04	Captive	Rietvlei NR	Unexposed	M	SA	Negative	Negative	Negative	unlikely	Negative
NZG 05	Captive	Rietvlei NR	Unexposed	F	SA	Positive	Negative	Negative	unlikely	Negative
NZG 06	Captive	Rietvlei NR	Unexposed	F	SA	Positive	Negative	Negative	unlikely	Negative
NZG 07	Captive	Rietvlei NR	Unexposed	M	AD	Positive	Negative	Negative	unlikely	Negative
NZG 08	Captive	Rietvlei NR	Unexposed	F	AD	Negative	Negative	Negative	unlikely	Negative
NZG 09	Captive	Rietvlei NR	Unexposed	F	AD	Positive	Negative	Negative	unlikely	Negative
NZG 10	Captive	PTA Zoo	Unexposed	M	AD	Negative	Negative	Negative	suspect	Negative
NZG 11	Captive	PTA Zoo	Unexposed	F	AD	Negative	Negative	Negative	probable	Negative
NZG 12	Captive	PTA Zoo	Unexposed	F	JU	Positive	Negative	Negative	suspect	Negative
NZG 13	Captive	PTA Zoo	Unexposed	F	JU	Negative	Positive	Negative	suspect	Negative
KNP40	Captive	PVT	Unexposed	M	AD	Negative	Negative			Negative

Lion #	Origin	Pride	*M. bovis* exposure	Sex	KNP Age class	Skin-test	STAT-pak	BAL	QFT GEA classification	FIV
KNP41	Captive	PVT	Unexposed	F	AD	Negative	Negative		unlikely	Negative
U03	Captive	Ukutula	Unexposed	M	SA				unlikely	
U04	Captive	Ukutula	Unexposed	M	AD	Negative	Negative	Negative		Negative
U05	Captive	Ukutula	Unexposed	M	AD	Negative	Negative	Negative		Negative
U06	Captive	Ukutula	Unexposed	M	SA	Negative	Negative	Negative	probable	Negative
U09	Captive	Ukutula	Unexposed	M	SA	Positive	Negative	Negative	unlikely	Negative
U10	Captive	Ukutula	Unexposed	M	SA	Positive	Negative	Negative	suspect	Negative
U11	Captive	Ukutula	Unexposed	F	JU	Positive	Negative	Negative	unlikely	Negative
U12	Captive	Ukutula	Unexposed	F	SA	Positive	Negative	Negative	unlikely	Negative
U13	Captive	Ukutula	Unexposed	F	AD	Positive	Negative	Negative	probable	Negative
U15	Captive	Ukutula	Unexposed	F		Positive	Negative	Negative	unlikely	Positive
U16	Captive	Ukutula	Unexposed	M	AD	Positive	Negative	Negative	suspect	Negative
U17	Captive	Ukutula	Unexposed	M	AD	Positive	Negative	Negative	unlikely	Negative
U18	Captive	Ukutula	Unexposed	M	AD	Negative	Negative	*M. terrae*	unlikely	Positive
U19	Captive	Ukutula	Unexposed	F	JU	Positive	Negative	Negative	unlikely	Negative

2.4 Discussion

Using antemortem diagnostic tests, that in many cases are not specifically validated for use in lions, to classify individual lions into different *M. bovis* infection or disease groups is not as straight forward as one would hope. Even in human subjects for which there is far more information, TB disease classification is complex and controversial. Human TB cases can be classified as follows:

- Definite - Culture and smear positive, and X-ray positive (by means of radiography of the lungs)
- Probable – When no culture was done but positive X-ray results and maybe a positive smear result was observed. Additionally, the individual needs to show improvement with TB treatment.
- Possible – When X-rays show possible but not definitive positive results together with a smear positive result but has a poor response to TB treatment.
- No TB - No evidence for TB with the above mentioned tests. Adults may still have positive skin test and IFN-γ result. However, for young children, positive IFN-γ and skin test results associated with suspicious clinical signs may lead to a probable diagnosis (Personal communication, Prof. P. D. van Helden, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/SAMRC Centre for TB Research, Stellenbosch University).

No proper adequately powered controlled studies or follow-up in lions has been done to try to ascertain the end result or outcome of lions with different diagnostic status. Therefore, the paucity of knowledge with regards to the antemortem diagnosis of TB in lions make it difficult, in most cases, to draw definitive conclusions about the state of *M. bovis* infection and/or disease in the different sampled lion populations. In this study, the BAL culture test could be considered a direct and definitive means of diagnosing infection and disease as it samples viable *M. bovis* bacilli shed in the respiratory tract. The other three diagnostic test utilised in this study could be considered indirect diagnostic tests since all of them rely on some form of an immune response to generate a result. Although the main aim of this part of the study was to use these diagnostic tests to classify the lions into different disease groups, the findings of each diagnostic test will first be discussed independently before trying to draw this together in to a holistic result.

2.4.1 BAL culture diagnostics

Considering that in a variety of species the shedding of mycobacteria, such as *M. bovis*, in respiratory secretions is associated with active pulmonary disease (Miller et al. 2015), it is possible to say with relative certainty that in the current study at least one lioness from the KNP had active pulmonary TB due to *M. bovis*. This, however, does not exclude the possibility that other lions were experiencing active pulmonary TB. Infection in this lioness was established through culture methods using BAL samples.   Using this method, Miller et al. (2015) identified that 6% of the lions

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tested was shedding viable *M. bovis* through respiratory secretions. The lower prevalence (2.6% for KNP lions) identified in the current study could be due to variance from small numbers and the study population, which included both infected and uninfected lions since no necropsies were done to confirm infection or disease. As suggested by Miller et al. (2015) it is possible to miss *M. bovis* infected animals with this method due to expected intermittent shedding of viable organisms, decreased recovery of shed organisms due to the inability to draw a sample from the entire lung, and loss of viability associated with sample handling and transportation. Additionally, pulmonary tuberculosis is more often associated with advanced cases of disease (Viljoen et al. 2015). It would, therefore, be possible to misdiagnose animals that are sub-clinically diseased and/or not experiencing pulmonary tuberculosis. The 2.6% prevalence in KNP lions established by BAL sampling in this study is therefore likely to be an under-estimate of the actual infection and disease prevalence.

In addition, the BAL culture diagnostic method identified one non-mycobacterium infection (no species data available), and two NTM infections (*M. terrae and M. intracellulare*).

The *M. terrae* infection was identified in a male lion from Ukutula that was FIV positive. None of the other diagnostic tests came up positive in this lion. *M. terrae* has previously been identified in buffalo and/or cattle (Gcebe et al. 2013) or their natural environment (Gcebe et al. 2013; Michel et al. 2007) in South Africa. However, this is likely the first known report of an *M. terrae* infection in a captive lion. Although considered to be non-pathogenic, *M. terrae* infections in humans can result in debilitating disease (Smith et al. 2000). In the review by Smith et al. (2000) the upper extremities were the sites of the majority of infections caused by skin wounds. The second most infected organ was the lungs (Smith et al. 2000). With *M. terrae* being an environmental NTM, contamination of the BAL sample is also a possibility. However, the lack of other *M. terrae* diagnoses for the other lions processed at the same site and sampled with the same protocol reduces the likelihood, although not disproving, that this case was due to environmental contamination of the BAL sample. It should be noted that this male lion died of septicaemia early in 2015 but the identification of the causative agent was not possible owing to lack of timely access to the animal.

M. intracellulare forms part of the *M. avium* complex (MAC) group of organisms and has previously been isolated in bovids and their natural environment in South Africa (Gcebe et al. 2013). In humans, MAC infections are most often associated with pulmonary infections in patients with pre-existing underlying lung conditions (Falkinham III et al. 2001; Guthertz et al. 1989).

Both *M. terrae* and *M. intracellulare* are environmental NTMs, therefore, contamination of the BAL samples should not be excluded.

2.4.2 Intradermal tuberculin test (ITT)

The ITT is the only immune response based diagnostic test utilised in the current study that has been validated in wild lions. According to the ITT results the prevalence of *M. bovis* (or *M.*

tuberculosis complex (MTC)) infections in wild or free-ranging KNP lions is 77.8% and is 50% in captive lions. This is very high considering that in the high prevalence zone in the KNP a *M. bovis* prevalence of up to 36% (Miller et al. 2012) and 40-60% (Ferreira & Funston, 2010) were reported for buffalo, a key prey species for lions. The prevalence established in this study for KNP lions is similar to results obtained by Keet et al. (2000), namely 78.2% for lions originating from the area of high *M. bovis* prevalence in buffaloes. This then leaves us with the question of whether the prevalence reported for captive lions is true or not. If the *M. bovis* or MTC infection prevalence of 50% for captive lions is a true representation of the current situation in South Africa, it would mean that the problem of MTC or *M. bovis* infection for South Africa is much worse than currently believed. It would mean that, unbeknown to managers of captive wildlife species, *M. bovis* infections has spread into highly controlled environments previously unaffected by this disease. This is, however, highly unlikely. Considering the specificity (81.2%) of the ITT and the high prevalence of NTM infections in lions reported by Keet et al. (2010), and detected also in this study, it is very likely that data for the current study includes some false positive results. Alternatively, the possibility exists that due to the high prevalence of *M. tuberculosis* in the South African human population, that captive lions have been exposed to *M. tuberculosis* through contact with human handlers or keepers.

Another aspect to consider is the actual measurement of the inflammatory response and the subsequent process of assigning a positive or negative result. Firstly, the inflammatory responses in the KNP lions were measured with a spring loaded TB calliper, thereby reducing the possibility of measurement errors due to variations in applied pressure on the skin fold. In contrast, all the responses in the captive animals were measured with a standard sliding calliper and the amount of pressure exerted on the skin fold could therefore vary between measurements and between animals. Therefore, the possibility exists that some of the positive ITT results for the captive/*M. bovis* unexposed animals could be ascribed to measurement errors. By looking at the actual skin fold thickness increase it might be possible to gain more insights. Keet et al. (2010) reported a mean skin fold thickness increase at respectively the Bovine PPD and Avian PPD site of 4.39 ± 2.34 mm and 0.68 ± 1.38 mm for *M. bovis* infected lions, 4.5 ± 2.67 mm and 1.19 ± 1.62 mm for *M. bovis* co-infected with NTM, and 0.8 ± 1.11 mm and 0.51 ± 0.99 mm for NTM infected only. Results for the current study (Table 2.1) showed mean inflammatory responses for KNP ITT positive lions at the Bovine PPD injection site that were slightly greater than previously described by Keet et al. (2010). The inflammatory response at the Bovine PPD injection site of ITT positive lions from the *M. bovis* unexposed population (2.81 ± 0.76 mm) was less than that of wild/exposed ITT positive lions reported by Keet et al. (2010) but greater than that of only NTM infected lions. Interestingly, all of the inflammatory responses at the Avian PPD injection site of ITT positive lions were greater in the current study than previously described for *M. bovis* infected KNP lions (Keet et al. 2010). This, together with the fact that the inflammatory response at the Bovine and Avian PPD sites of ITT positive lions differed significantly in the KNP only, might suggest that the high

prevalence observed in captive lions is due to specificity problems of the ITT. Additionally, a possible flaw of the ITT could be the assumption that skin fold thickness is standard in all lions. The basis of the ITT is that a greater immune response to the antigens present in the Bovine PDD will lead to greater inflammation at the injection site of *M. bovis* infected animals than for uninfected animals. Initial skin fold thickness measurement in the current study ranged from 4 mm to 12 mm at the Bovine PPD site. A positive ITT result for a 4 mm thick skin required at least a 50% increase in skin fold thickness while for a 12 mm thick skin an increase of only 16.7 % was needed. Therefore, comparatively less proportional inflammation was needed in thicker skinned animals than in thin skinned animals to give a positive ITT result. This opens the possibility that slight immune response in a sensitised animal with a generally thicker skin might result in a false positive result. In the current study, initial skin thickness did not affect the likelihood of identifying animals as *M. bovis* positive. However, when the percentage increase of skin fold thickness was calculated[3] (data not shown) the percentage increase at the Bovine PPD site in wild ITT positive lions were approximately three fold greater than the percentage increase in captive ITT positive lions. Additionally, the percentage increase was significantly different between the Bovine and Avian PPD injection sites for only wild ITT positive lions. No differences were observed between the different locations for the percentage increase at the Avian PPD injection site.

Taking all of the above into consideration it is likely that the high prevalence of ITT positive captive lions, and therefore prevalence of *M. bovis* in captive lions, could possibly be explained by classifying these ITT results as false positives. If this is the case then the current study could also serve as an indication that the ITT parameters need to be re-assessed or adapted for captive lion populations. Additionally, the current findings also suggest that, especially for captive lions, the result at the Avian PPD site needs to be considered. This is further emphasised by the stronger correlation of Bovine and Avian PPD data for captive/*M. bovis* unexposed lions when compared to wild/*M. bovis* exposed lions (Figure 2.1). This stronger correlation between inflammatory response measurements at the Bovine and Avian PPD injection sites of captive lions compared to that in wild lions might serve as an indication of a more pronounced presence of MTC species other than *M. bovis* in the captive lion population resulting in false positive ITT results.

2.4.3 TB Stat-Pak

Keet et al. (2010) suggested the use of serology based diagnostic tests in concert with the ITT to increase the sensitivity of the ITT. The study by Miller et al. (2012) was able to identify 64% of known infected lions using the serologic TB Stat-Pak test. Considering the TB Stat-Pak results in concert with ITT results they identified 73% of infected lions in contrast to only 63% using only the ITT. Unfortunately their study did not establish a TB Stat-Pak positive *M. bovis* prevalence for the general Southern-KNP lion population nor were they able to establish sensitivity and specificity (Miller et al. 2012).   The seroprevalence for KNP lions in the current study (8%) is double the 4%

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[3] Percentage increase = ((Skin fold measurement at 72h – initial skin fold measurement)/initial skin fold measurement) x 100

seroprevalence reported for Tanzanian lions (Cleaveland et al. 2005). This is to be expected since the prevalence of *M. bovis* in prey species is relatively high in the Southern part of KNP (Ferreira & Funston, 2010; Miller et al. 2012). The prevalence established for captive lions is similar to that previously described for Tanzanian lions. However, only one captive lion was TB Stat-Pak positive and this animal had a negative ITT result. This, together with the fact that the TB Stat-Pak makes use of antigens specific to MTC and not exclusively to *M. bovis,* raises the possibility of cross-reactivity and therefore a false positive result. All of the KNP Stat-Pak positive lions were ITT positive with one lion confirmed *M. bovis* positive through BAL culture diagnosis. Miller et al. (2012) proposed some limitations of the TB Stat-Pak test, suggesting that immature or compromised immune systems due to either advanced or very young age, severe disease, concurrent viral or parasitic infections, anergy, or therapeutic interventions could lead to false-negative results. Considering these limitations it might be that the current TB Stat-Pak prevalence for KNP lions is likely to be an under-estimate.

2.4.4 QFT gene expression assay (GEA)

The GEA has not been validated for lions nor are sensitivity and specificity data available. However, Olivier et al. (2015) were able to distinguish between lion populations with different suspected *M. bovis* and MTC exposure pressures and subsequently proposed different cut off values to group lions as either probably infected, suspected of being infected or unlikely to be infected. These cut off values are not set in stone and could likely change as more animals are tested with the GEA (Olivier et al. 2015). Therefore, using the GEA alone, it is not possible to definitively classify a lion as *M. bovis* infected or not. However, in a more recent study (Sylvester et al. 2016) using the cut off value of 27 to distinguish between *M. bovis* infected and uninfected wild lions managed to show differences in *M. bovis* prevalence between different regions in the KNP that were similar to estimated *M. bovis* prevalence in buffalo (Sylvester et al. 2016). They described a prevalence of 54% in the southern regions of the KNP (Sylvester et al. 2016). This is of the same order as the 48% prevalence (Table 2.2) described for probably infected (by means of the GEA) KNP lions from the same area in the current study.

As could be expected, results from the current study indicated that a greater proportion of wild/*M. bovis* exposed lions were, according to the proposed cut off parameters, probably infected or suspected of being infected while a greater proportion of captive/*M. bovis* unexposed lions were unlikely to be infected. This could possibly explain the significant differences in GEA responses (Figure 2.2) observed between KNP/*M. bovis* exposed lions and the captive/*M. bovis* unexposed lions. Interestingly a relatively high percentage of captive lions were suspected of being infected with some lions identified as probably infected. As mentioned before, the possibility exists that the captive lions more frequently than wild lions might be exposed to MTC bacilli other than *M. bovis*. This, together with the fact that for the GEA, blood is stimulated with peptides simulating MTC

antigens not specific to *M. bovis* alone, might indicate some form of cross-reactivity with other current or past MTC infections.

2.4.5 FIV

Keet et al. (2010) established that although FIV infections could possibly affect the outcome of immune based diagnostic results, in their study it did not affect the ITT results. The current study established a 100% FIV prevalence (n = 39) in KNP lions (Table 2.4) compared to 47% (n = 34/72) seroprevalence described for the Keet et al. (2010) study group. It should be noted that lions in the current study only represented the southern part of the KNP's lion population whereas the Keet et al. (2010) study consisted of lions sampled throughout and along the periphery of the KNP. In the current study a variety of *M. bovis* diagnostic results were obtained for the wild/KNP FIV positive lions (Tables 2.3 and 2.4). This might suggest that FIV infection does not affect the diagnostic tests used in this study.

The FIV diagnostic test used for the captive lions was developed for domestic cats. Some of the lentiviruses infecting wild felids have been reported to show some genetic differences to that of domestic cats (van Vuuren et al. 2003). Therefore, these tests may not have the same specificity or sensitivity for retroviruses of wild felids (van Vuuren et al. 2003). The small percentage of FIV positive captive lions might therefore be an under-estimate. However, three of the captive FIV positive lions were housed together, with these results conforming to the highly infectious nature of FIV. While this could be an indication that the IDEXX® Snap test can be used for lions the small sample size (n = 5) of FIV positive captive lions prevents definitive conclusions to be drawn.

2.4.6 *M. bovis* diagnosis in relation to age and sex

The question regarding the effects of TB on lion social and population dynamics still needs to be properly addressed. Lions are a social and aggressive species. Alterations to the normal roles and functioning of the different sexes and age groups have the ability to affect the survival of individuals as well as whole prides. By looking at the age and sex of lions that are more likely to be *M. bovis* infected or experiencing active disease it is possible to start to get some insights into the threat of TB to lions.

Assuming at this point that the diagnostic results for KNP lions are representative of *M. bovis* infection, the following can be concluded. The age related differences in diagnostic results of the KNP lions are to a large part similar to what was described by Keet et al. (2000) and Miller et al. (2012). Keet et al. (2000), using the ITT, found that adults were more likely to be infected than sub-adults or cubs. Similarly, Miller et al. (2012) found that the majority of culture positive animals were adults and that only lions older than 2.5 years of age had a TB Stat-Pak positive result. However, the youngest ITT positive lion was seven months old (Keet et al. 2000) and culture positive lion was almost 10 months old (Miller et al. 2012). In the current study the youngest animals with ITT positive results were two 4-6 month old KNP lions while the youngest animal with a positive TB Stat-Pak result was a 1.5 year old captive NZG lion. Additionally, the current study

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found that a greater percentage of female lions were either ITT positive or TB Stat-Pak positive. This is different to the proposition that male lions may be at greater risk of infection due to fighting, movement between prides, and social hierarchy during feeding (Keet et al. 2009). Similarly, the gender based differences for GEA results of wild lions in the current study also differed from that reported by Sylvester et al. (2016) where in their study a trend (not significant) of higher prevalence in male lions compared to female lions were observed. In the current study, by means of the GEA, wild female lions showed a significant higher prevalence than male lions. A possible explanation for these differences between this study and that of Sylvester et al, (2016) conducted during the same time frame on lions from the same area could possibly be ascribed to relative small sample numbers in both these studies. The age related GEA responses in this study were similar to that described in Sylvester et al. (2016) in that higher prevalence of *M. bovis* probably infected cases were reported for wild adult lions compared to juvenile lions. The slight difference in values between this study and that of Sylvester et al. (2016) might be ascribed to the age classification system used where Sylvester et al. (2016) differentiated between immature (<4 years) and mature (≥ 4 years) lions. In addition to their suggestion that the age related differences could be due to increased exposure to infected prey over time for mature lions or alternatively the possibility of increased mortality of *M. bovis* infected younger lions (Sylvester et al. 2016), the current age related GEA differences may serve as an indication of the chronic nature and age dependant progress of the disease with juveniles suspected of being infected converting to probably infected as they grow older. .

Considering all of this, it would seem that in the current study adult female lions are more likely to be *M. bovis* infected or experiencing disease. The implications of this will be discussed in more detail in Chapter 7.

The age related differences observed in the captive lions were to a large part the reverse of what was seen in wild lions. The chronic nature of *M. bovis* infections and/or disease can also not account for these differences. Differences between the sexes were similar to that seen in wild lions. One possible explanation for these findings might be that captive lions were exposed to *M. bovis* due to human error, if for example, unknowingly contaminated carcasses were fed to the lions. Alternatively, these findings might be a further indication of a lower specificity for diagnostic tests in captive, publicly kept lion populations. Considering that many of the diagnostic tests rely on an immune response to MTC antigens and not *M. bovis* specific antigens, this becomes a factor in captive lion populations that possibly are more readily exposed to MTC species other than *M. bovis* compared to free-ranging lions (See section 2.2.1).

2.4.7 Multiple diagnostic results

The previous sections looked at the results of each of the diagnostic tests independently. Due to the paucity of information available for the different diagnostic tests used in this study, it is not possible to make definitive conclusions regarding differentiation between *M. bovis* infections or

active disease in lions. A better idea of the state of *M. bovis* infection/disease in lions might be formed when one consider the multiple diagnostic results simultaneously while taking into account the origin of the sample. This may also aid in describing the usability of the different tests on captive lion populations. In the current study a total of 66 lions were tested with three or more diagnostic tests. Looking at the results from diagnostic tests that give only a positive or negative result (i.e. ITT, TB Stat-Pak, and BAL) none of the captive lions had concurrent positive diagnoses. Four out of six captive lions that were identified as suspected of infection by means of the GEA had concurrent positive diagnoses. Of the three captive lions that were identified as probably infected, by means of the GEA, only one lion had an additional positive ITT result while none of the other diagnostic tests were positive for these three lions. For the wild lions all the TB Stat-Pak positive diagnoses were accompanied by a positive ITT diagnosis. Additionally, the majority of ITT positive diagnoses had a concurrent GEA suspected or probably infected classification. Assuming that the captive lions were not exposed to *M. bovis*, these findings might indicate a poor specificity of these diagnostic tests in captive lion populations, specifically for the ITT.

Since no necropsies were performed to confirm infection or disease, any further assumptions will be purely speculative. The probability of infection classification of lions by means of the GEA is reliant on the MIG (monokine-induced by gamma interferon) gene production response in lion blood stimulated with MTC antigens (Olivier et al. 2015). MIG is produced in response to IFN-γ (Gasperini et al. 1999). The greater MIG gene production, suggesting increased IFN-γ (Gasperini et al. 1999), in lions classified as probably infected by means of the GEA compared to lions classified as suspect of infection together with the important role of IFN-γ in the CMI response to TB (de la Rua-Domenech et al. 2006; Tebruegge et al. 2015) allows one to speculate that when a wild/*M. bovis* exposed lion has a concurrent ITT positive and GEA probably infected diagnosis it is possibly experiencing a more advanced CMI response and therefore sub-clinical disease when compared to an animal with a concurrent ITT positive and GEA suspected infection diagnosis. The KNP lion from which the *M. bovis* positive BAL sample was obtained was both ITT and TB Stat-Pak positive. It is, therefore, reasonable to assume that the other two KNP lions that were ITT and TB Stat-Pak positive and had a probably infected GEA classification were indeed experiencing active disease.

The presence of NTM species might affect the outcome of some diagnostic tests such as the ITT (Keet et al. 2010). Assuming that the *M. terrae* diagnosis was due to exposure or infection and not contamination, the lack of accompanying positive diagnoses by means of the other tests might serve as an indication that this species of NTM does not cause false positive *M. bovis* diagnostic results. This does not seem to be the case for *M. intracellulare,* since all other diagnostic tests came up positive together with a probably infected GEA classification. However, *M. bovis* infections in lions are not limited to pulmonary infections and various limitations are associated with BAL sampling (see BAL discussion in section 2.1 and section 2.4.1). Factoring in that this lion was an adult female living in an area with known *M. bovis* exposure it is possible that

this lion was infected with both *M. bovis* and *M. intracellulare* with only the latter being sampled by means of the BAL. Cross-reactivity of *M. intracellulare* with the utilised diagnostic tests, although unlikely, cannot be conclusively excluded.

2.5 Conclusion and classification of study groups

The hypothesis presented in chapter one (section 1.3) that - "The use of multiple antemortem diagnostic tests will reduce the need for necropsy in order to classify the studied lions into cohorts of different *M. bovis* disease and/or infection categories" – was partly disproven in this chapter. For reasons presented earlier in the discussion section (section 2.4) and in the following paragraphs the hypothesis should rather be changed to the following statement: "In the absence of post-mortem diagnostics it is possible to use multiple antemortem diagnostic tests in concert to classify a subset of sampled lions into at least two categories of suspected infection status". Note the use of the word "suspected" and the exclusion of "disease status" since at this point it is still not possible to get a definitive diagnosis without post-mortem diagnostics.

This study showed that the antemortem diagnosis of *M. bovis* in lions is more complicated than purely relying on the positive or negative results obtained from diagnostic tests. Diagnostic results for the wild/KNP lions were similar to that previously described for KNP lions. However, at first glance the initial diagnostic results showed an unexpected high *M. bovis* infection rate of captive lions that should theoretically not be exposed to *M. bovis*. However, when the quantitative data of the diagnostic tests were compared and the all the diagnostic results were considered the possibility emerged that the specificity of the diagnostic tests are reduced in captive situations. A possible explanation for this would be the higher suspected exposure of captive lions to MTC bacilli other than *M. bovis*. Considering the findings of this study and with the available knowledge, interpretation of the antemortem diagnostic results should likely be left to the discrimination of the investigator. This interpretation should be guided by knowledge of suspected MTC exposure other than *M. bovis* through contact with or proximity to other species, including humans, which could be carriers of MTC species, knowledge of the life history of each animal with regards to housing history, management practices, as well as medical history if available. Furthermore, an approach similar to what is seen in human cases (as described in the introductory paragraphs of section 2.4) might be needed to aid in infection/disease classification.

One of the reasons for this investigation into the use of multiple antemortem diagnostic tests was to get an idea of the *M. bovis* infection and/or disease status of the lions sampled in order to facilitate a comparative, investigative study into the possible immune/inflammatory, energy metabolism and reproductive endocrine effects of *M. bovis* on lions. Due to the lower suspected specificity of diagnostic tests in captive lions and the paucity of information on the sensitivity and specificity of these tests in wild lions, it was not possible, with certainty, to classify lions beyond reasonable doubt into different classes without performing a necropsy. However, by applying stringent selection criteria it might be possible to classify a subset of the lions sampled in the

current study into two types of infection groups from which to compare metabolic markers. The two classifications with their selection criteria are:

- Very likely infected with *M. bovis* (probably infected) – The lion must live in an area with known *M. bovis* exposure; one or more of the ITT, TB Stat-Pak, and BAL tests must be positive and the GEA classification either suspect or probably infected; if no GEA data are available and two or more of the other diagnostic tests are positive, that lion is also classified as probably infected.

- Very unlikely to be infected with *M. bovis* (uninfected) – If a lion lives in an area with known *M. bovis* exposure, none of the ITT, TB Stat-Pak, or BAL tests must be positive and classified as unlikely infected by means of the GEA; lions living in an area with no known *M. bovis* exposure, had two or more diagnostic tests done with all tests coming up as negative; any lion with a GEA classification of probably infected are excluded from this classification.

Using these criteria it is assumed that lions with false positive diagnoses as well as possible latently infected[4] lions will be excluded from the likely *M. bovis* infected group. These criteria will likely exclude lions that could possibly be infected with MTC bacilli from the uninfected classification. FIV status was not considered during selection.

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[4] To my knowledge latency has not been proven in lions. It has however been suggested as a possible infection state in (Keet et al. 2009). Additionally, it was described to a degree by Eulenberger et al. (1992) as the state of infection preceding the onset of clinical signs due to environmental stressors (Maas, 2008).

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Chapter 3

Stress responses and immune/inflammatory markers associated with *M. bovis* infections in lions

3.1 Introduction

Infections and diseases elicit an immune response aimed at eliminating the infection or disease from the body. The type of immune response is related to the type of infection or disease and can range from an acute localised reaction to a chronic systemic response facilitated by either the innate or acquired immune systems or both (Fearon & Locksley, 1996). The type of immune response and the different phases of the response are regulated by different subdivisions of the immune system (Fearon & Locksley, 1996). Knowledge of the type and phase of response and immune system subdivisions active in the fight against an infection or disease can be important for a number of reasons, for example: a) to gain a better understanding of the pathogenesis of the disease; b) to develop diagnostic procedures, c) to develop treatments for the disease; and d) to better understand disease epidemiology. Tuberculosis due to *Mycobacterium bovis* infections in lions is one such disease that is in need of better understanding.

Not much knowledge is available about the immune response of lions towards an *M. bovis* infection. Additionally, the complex interplay of the immune system with metabolic pathways and the possibility that one system may have an effect on the function of other systems has not been studied in lions infected with *M. bovis.* For instance, biological markers such as cortisol and CRP (C-reactive protein) produced in response to disease stress and inflammation might impact on energy metabolism as well as the reproductive endocrine system. Since not much knowledge is available regarding specific lion immune responses to *M. bovis* infection or disease some insights might be drawn from other mammalian species' responses to *M. bovis* that could serve as a basis from which to study immune/inflammatory responses to *M. bovis* in lions.

During an immune/inflammatory response to an infection or disease, the body produces a variety of biological markers that serve as messengers within the body or are by-products from the processes active in fighting the infection (Fearon & Locksley, 1996; Pollock et al. 2001; Rook, 1999; Santucci et al. 2011). By measuring these markers, insights can be gained about the type of immune response, the type of immune/inflammatory processes, and possible secondary effects caused by the immune/inflammatory response. The main aim of this chapter will be to initiate an investigation into some of these immune/inflammatory and stress markers in the presence of suspected *M. bovis* exposure and/or infection in lions. For the purpose of the current study the markers that will be investigated are various cytokines, cortisol and CRP. More details on these markers are included under their representative sections in this chapter. In this context, this work may be regarded as hypothesis generating, rather than hypothesis driven: i.e. it may be stated that

it is hoped we may be able to find biomarkers that can be used to investigate stress owing to *M. bovis* infection in lions. This is the approach taken in much recent biomarker work in humans (Chegou et al. 2016; Jacobs et al. 2016a; Jacobs et al. 2016c; Jacobs et al. 2016b).

The focus of this chapter will be guided by the following objectives or questions. Firstly, establish if it is possible to measure the above mentioned biomarkers in lion samples by making use of easily obtainable commercially available assays or laboratories? Secondly, if usable data can be obtained, is it possible to distinguish between different cohorts of lions with different *M. bovis* exposure pressures or suspected infection statuses? Thirdly, identify possible relationships between the different biological markers that could potentially indicate unexpected interactions, confirm usability of the assays, and/or give new insights into immune/inflammatory responses of lions to *M. bovis* that could aid the direction of future research.

3.1.1 Cytokines

Simplified, the types of immune response can be roughly grouped into type 1 and type 2 responses with different subsets of immune cells active in each type of response. Type 1 responses are primarily mediated by macrophages that involve phagocytosis and intracellular killing of microorganisms. Type 2 responses are macrophage-independent, mediated by non-cytotoxic antibodies, mast cells, and eosinophils (Fearon & Locksley, 1996). In this regard, helper T cells (TH) are also divided into subsets of TH-1 and TH-2.

Cytokine is a collective name given to a variety of proteins produced during these immune/inflammatory responses that serve as messengers to guide the different types and phases of the immune response. For example, TH-1 cells promote type 1 immune responses by secreting cytokines such as IFN-γ, lymphotoxin, and TNF-α that increase the microbicidal activity of macrophages. TH-2 cells mediate type 2 responses by secreting cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13 which collectively mediate the growth and activation of mast cells and eosinophils, and inhibit macrophage activation (Fearon & Locksley, 1996).

An example of how knowledge of the cytokine profiles can be used to gain insights into disease can be seen in the study by Chen et al. (2016). They were able to identify characteristic cytokine profiles in human patients at different time points during the course of TB disease due to *M. tuberculosis*. Interpretation of the cytokine profiles allowed them to gain more insights into the disease pathologies at the different disease states as well as how the host's immune system interacted with the disease at the different time points (Chen et al. 2016). Additionally, the cytokine profiles of latently infected patients differed significantly from that of cured patients that had active TB, possibly indicating a contradiction to the traditional belief that cured active disease patients are identified as latently infected (Chen et al. 2016). However, they did mention that this difference in cytokine profiles might have been due to the chemotherapy treatment these patients received (Chen et al. 2016). Furthermore, a plethora of recent publications showed the value of cytokines and cytokine profiles as diagnostic tools for TB in humans as well as the ability to monitor the

disease treatment response of patients with samples obtained from a variety of bodily fluid sources such as whole blood (Sutherland et al. 2016), pleural effusions (Sutherland et al. 2016), saliva (Jacobs et al. 2016a; Jacobs et al. 2016c), serum (Chegou et al. 2016), and plasma (Jacobs et al. 2016b).

The study by Boddu-Jasmine et al. (2008) investigated the expression of cytokine mRNA in lymph node samples from cattle experimentally infected with logarithmically increasing doses of *M. bovis* at 26 weeks post-infection. They specifically looked at the pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokines believed to play an important part in the host's ability to fight infection. All the cytokines except TNF-α showed a significant increase in relation to increased *M. bovis* infection dose. The insignificant increase of TNF-α was considered to be due to the role of this cytokine in the initial innate immune response or alternatively due to regulation of this cytokine by anti-inflammatory cytokines such as IL-4 and IL-10 (Boddu-Jasmine et al. 2008). They suggested that lower cytokine production might be needed to prevent disease in cases of lower doses of *M. bovis* infection and higher cytokine production for higher dose infections (Boddu-Jasmine et al. 2008). Interestingly, Boddu-Jasmine et al. (2008) reported that despite significant differences in cytokine expression associated with increased infection doses (1 cfu vs.10 cfu vs.100 cfu vs. 1000 cfu) the resultant pathology was similar irrespective of initial *M. bovis* infection dose.. Additionally, they mentioned previous studies that reported low levels of the anti-inflammatory cytokines IL-4 and IL-10 during the initial stages of disease with increases only at later stages. This was ascribed to early disease stages likely being dominated by Th-1 type responses with IFN-γ being the predominant cytokine that, as the disease progresses, shifts to a combined IFN-γ and IL-4 mediated response and subsequently an IL-4 dominated Th-2 response (Boddu-Jasmine et al. 2008).

An earlier study in cattle looking at the gene expression over time of the same cytokines, but also including iNOS, in PBMC and lymph nodes close to the site of infection was done by Thacker et al. (2007). In contrast to some of the statements made by Boddu-Jasmine et al. (2008), they found that IFN-γ, TNF-α, iNOS, and IL-4 production by PBMC increased in response to infection while IL-10 production decreased (Thacker et al. 2007). They found that the peak for the Th-1 response in cattle was at 30 days post infection with a decline as infection progressed. The magnitude of this early Th-1 response was positively correlated with the degree of pathology present later (Thacker et al. 2007). Interestingly they also found that cytokine gene expression at later stages of infection differed between animals with extensive or low level pathology. The PBMC of animals with extensive pathology, when stimulated with rESAT6:CFP10, continued to express similar or slightly increasing levels of cytokine mRNA while animals with low level pathology expressed less. In contrast, when RNA was isolated from lymphoid tissue adjacent to lymph lesions, animals in the low level pathology group expressed more IFN-γ and iNOS than animals with extensive pathology (Thacker et al. 2007). Additionally, IL-10 mRNA expression was reduced in cattle with more severe pathology (Thacker et al. 2007). In this study they suggested that for

cattle, a shift from Th-1 to Th-2 type responses does not occur during the first three months after infection (Thacker et al. 2007).

These studies of cytokine response in cattle infected with *M. bovis,* together with *M. tuberculosis* studies in mice and humans gives an indication of the complexity associated with cytokine function and expression, even for well studied species especially when time, route and dose of infection is known.

From the above it might be easy to assume that knowledge of individual cytokine expression is not of much use when the time and dose of infection is unknown. The type of sample must also be taken into consideration when interpreting cytokine data, since different tissue types and proximity to the site of infection or lesions could also affect the cytokine expression profile. However, more recently various studies have suggested that protein biomarker concentrations may discriminate between healthy humans and patients with active TB, and latent TB. For example, Essone et al. (2014) conducted a pilot study to evaluate the use of antigen-induced biomarkers in short-term antigen stimulation assays for the diagnosis of active TB in humans. None of the antigen-induced markers were able to individually discriminate between active TB and non-TB cases with all receiver operator characteristic (ROC) analyses yielding area under the curve (AUC) values of less than 0.85. However, when these markers, together with unstimulated acute phase protein markers (CRP and SAA) were incorporated into four-marker models, some of the combinations had a sensitivity and specificity of more than 85%. This was despite the small sample size (n = 30) in this pilot study. Single markers that showed potential as diagnostic tools were antigen specific concentrations of MMP-9, EGF, IP-10, and unstimulated concentrations of CRP and SAA (serum amyloid A). The unstimulated acute phase proteins (CRP and SAA) together with the cytokines TGF-α, VEGF, EGF, and IFN-α2 featured strongly in the top four-analyte multi-marker models capable of diagnosing TB in human patients (Essone et al. 2014).

Tebruegge et al. (2015) were able to discriminate between human children that were uninfected, latently *M. tuberculosis* infected or experiencing active TB. This was done by stimulating whole blood with ESAT6, CFP10 and PPD and measuring the cytokine responses. They found that the cytokines able to discriminate between uninfected individuals and latently infected or diseased individuals were IP-10, TNF-α, IL-1ra, IL-2, IL-13, and MIP-1β. The responses of TNF-α, IL-1ra, and IL-10 had the greatest ability to discriminate between latently infected and diseased individuals. Additionally, by combining the results of TNF-α and IL-1ra or TNF-α and IL-10, they could accurately discriminate between latently infected and diseased individuals. The TNF-α and IL-1ra or TNF-α and IL-10 combinations classified respectively 95.5% and 100% of the studied individuals correctly (Tebruegge et al. 2015). This study obtained a higher degree of discrimination than a similar earlier study by Frahm et al. (2011), that managed to accurately identify 83% of diseased patients and 88% of latently infected patients using a combination of IL-15 and MCP-1 cytokines. Major differences between these studies were the mean age of the study populations, the method used to stimulate the blood with antigens, and the fact that the Tebruegge

et al. (2015) study obtained samples from individuals that had not received any treatment, whilst in the Frahm et al. (2011) study, patients that had already initiated or completed treatment were sampled.

To my knowledge not much is available on *M. bovis* specific cytokine profiles for lions. Maas (2008) did a preliminary study to develop a real time qPCR for lion IFN-γ, TNF-α, IL-10 and IL-4 to use as a tool for investigating immune status during a *M. bovis* infection. In that study, ITT positive lions had almost a three-fold elevated expression of IFN-γ and approximately two-fold elevated expression of TNF-α than ITT negative lions (Maas, 2008). Differences in IL-10 expression were minimal while IL-4 was approximately four times lower in ITT positive lions than in ITT negative lions (Maas, 2008). However, infection status and disease severity was not substantiated by necropsy and Maas (2008) also aired caution in interpretation of these results due to the preliminary nature of the study and need for further optimisation and validation of the process.

The potential to make use of cytokine profiles in diagnosing TB, describing and understanding disease course and pathologies, and the lack of knowledge regarding cytokine profiles in lions exposed to, infected, or diseased with *M. bovis* calls for more research that will aid in furthering this line of investigation.

3.1.2 Cortisol

Cortisol is a steroid hormone produced by the adrenal gland when the hypothalamic-pituitary-adrenal (HPA) axis is stimulated by various pro-inflammatory cytokines produced at centres of infection and inflammation. Additionally, high stress situations also stimulate the HPA axis to produce cortisol (Rook, 1999).

The mechanisms regulating cortisol production, function, and the subsequent effects it might have on the immune system are complex. Although overall concentrations of cortisol might be increased in an individual, the effect of the cortisol can be site-specific. Various factors such as cytokines, type and concentration of different receptors in cells and tissue types, and enzymes play a role in the effect cortisol can have at a specific site (Rook, 1999). Additionally, in humans and rodents, diurnal cycling of circulating cortisol concentrations occurs, with rodents having an inverse cycle to that of humans (Rook, 1999). However, domestic cats do not seem to have a circadian cortisol rhythm (Nogueira & Silva, 1997).

Cortisol together with other anti-inflammatory cytokines has been shown to drive the immune response from a Th-1 type response to a Th-2 type response (Rook, 1999). In *in vitro* studies, Mahuad et al. (2004) found that cortisol within physiological and/or pathophysiologic ranges – such as during acute stress - inhibits cellular proliferation and IFN-γ production from PBMC stimulated with mycobacterial antigens. The PBMC of human patients experiencing advanced disease seem to be extremely sensitive to the inhibitory effect of cortisol on IFN-γ production (Mahuad et al. 2004). However, the effect of cortisol on lymphoproliferation of cells was

proportionally and significantly less in samples from human patients with advanced disease than samples from healthy controls or patient with mild or moderate disease (Mahuad et al. 2004). Additionally, cortisol did not have an effect on the production of the anti-inflammatory IL-10 cytokine. Both cortisol and IL-10 may play a role in facilitating Th-2 activity, partly by their inhibitory effects on Th-1 cells (Mahuad et al. 2004).

Cortisol can inhibit many macrophage functions, including some antimicrobial functions. However, the precise effects of cortisol on macrophages are dependent on dose and time of exposure. The inhibitory effects are also influenced by the presence or absence of specific cytokines, receptors, and enzymes. Additionally, not all antimicrobial functions are affected by cortisol and it depends on whether the relevant antimicrobial function is sensitive to glucocorticoids or not (Rook, 1999). Furthermore, the effects of cortisol on immune function are not solely limited to inhibition and under certain circumstances can also be stimulatory in nature (Rook, 1999).

The inhibitory effect of glucocorticoids is not limited to humans. The study by Brown et al. (1993a) found that activation of the HPA axis through restraint induced stress, made BCG susceptible mice more susceptible to mycobacterial growth. Additionally, activation of the HPA axis suppressed the ability of macrophages to produce TNF-α after stimulation with recombinant IFN-γ and lipopolysaccharide (Brown et al. 1993a). In line with this inhibitory function of cortisol, Willemse et al. (1993) suggested that the reported weak skin test reactivity in domestic cats could possibly be explained by increased levels of cortisol in response to stress experienced during handling and skin testing.

From the above it is clear that the effects of cortisol on immune function differ between types of infection, and includes differences in susceptibility to and progression of disease. In this regard an altered cortisol to cortisone ratio biased towards cortisol was observed in human TB patients (Rook, 1999). Mahuad et al. (2004) suggested that in human TB cases, cortisol might play a role in restricting tissue-damaging immune responses usually accompanying cellular anti-mycobacterial immune reactions. Furthermore, an inhibitory role of cortisol on the ability of human and murine macrophages to control the growth of *M. tuberculosis* has been reported (Brown et al. 1993a; Mahuad et al. 2004; Rook, 1999).

Due to the complexity associated with cortisol responses to various factors, it is difficult to make predictions of what will be observed in lions. One might anticipate that if lions are experiencing a Th-1 type immune response to infection that the resultant increase in pro-inflammatory cytokines might result in increased cortisol levels.

3.1.3 CRP

C-reactive protein (CRP) is one of many acute phase proteins produced by the liver in response to an acute inflammatory stimulus. As with many other acute phase proteins, induction of CRP production by hepatocytes is principally regulated by IL-6 and can be enhanced by IL-1β.

Synthesis of CRP by other non-hepatic cells has been reported but is suspected to be unlikely to substantially affect plasma CRP levels (Black et al. 2004).

Murine *in vivo* studies identified a protective role of CRP against bacterial infections and suggested a net anti-inflammatory function, while *in vitro* studies have described context-dependant pro- or anti-inflammatory functions for CRP in humans (Black et al. 2004). Species differences between mice and humans have been reported in inflammatory CRP response levels, with the reduced murine response ascribed to an evolutionary oddity (Black et al. 2004).

Pro-inflammatory functions reported for CRP include: reacting with various ligands - some of which are present in a number of bacterial species - leading to the activation of the classical complement pathway; stimulation of phagocytosis through interactions with FcγR immunoglobulin receptors; up-regulating the expression of adhesion molecules in endothelial cells; inhibition of endothelial nitric-oxide synthase expression in aortic endothelial cells; increasing the release of IL-1, IL-6, IL-18, and TNF-α; stimulation of the release of IL-8 from various cell types; increasing plasminogen activator inhibitor-1 expression and activity (Black et al. 2004). On the other hand, the ability of CRP to induce the expression of IL-1 receptor antagonist, increase the production of the anti-inflammatory cytokine IL-10, and suppression of IFN-γ production is an indication of some of the anti-inflammatory roles of CRP (Black et al. 2004).

The individual components of the classical complement activation cascade leads to different levels of inflammatory responses, with later stage components being highly inflammatory. Studies that examined the individual components suggested that CRP facilitates activation of the earlier stages with little activation of the late components. This suggests that CRP participates in host defence systems while limiting the possibly damaging inflammatory responses associated with the later stage complement components (Black et al. 2004).

Relatively little data is available regarding CRP concentrations and tuberculosis. The study by van Crevel et al. (2002) reported significantly higher plasma CRP concentrations in human TB patients compared to healthy controls. They also found an inverse correlation of CRP to leptin concentrations (van Crevel et al. 2002).

A strong correlation was found between CRP and serum amyloid A protein (SAA) in human TB patients (de Beer et al. 1984). This study, which assessed concentrations of CRP and SAA, showed that there were different levels of acute phase responses associated with the different disease states. In this regard, patients with primary TB did not have a significant acute phase response while patients with post-primary TB without lung lesions showed a moderate response and patients with post-primary TB with lung lesions or with miliary TB had a major acute phase response (de Beer et al. 1984). Additionally, de Beer et al. (1984) established that once disease treatment commenced, the serum SAA and CRP levels decreased within two days. Interestingly, Kajikawa et al. (1999) found that in domestic cats, in contrast to human, equine, or canine models, CRP is not a highly responsive acute phase protein and that SAA is a responsive protein at early

stages of inflammation. Despite this, CRP concentrations in hospitalised cats (reasons for hospitalisation not given) were significantly ($p < 0.005$) higher than in healthy control cats (Kajikawa et al. 1999).

The *M. tuberculosis* vaccine study by Langermans et al. (2005) used CRP concentrations together with other biological markers to indicate the protective value of their proposed vaccine. In this study CRP concentrations correlated with disease severity (Langermans et al. 2005).

To my knowledge no information on CRP concentrations in lions is readily available. Therefore, considering that increased CRP levels were associated with miliary TB (de Beer et al. 1984), that human patients with active TB had higher CRP concentrations than healthy individuals (van Crevel et al. 2002), and that CRP has been used as a marker of chronic inflammation (Bottasso et al. 2013; Dandona et al. 2004; Gan et al. 2004), and assuming that species differences in CRP production is not present in lions compared to humans, then one might anticipate higher CRP concentrations in lions that are experiencing active TB due to *M. bovis* than in healthy lions.

3.2 Materials and Methods

It should be noted that there are no, or very few, biological assays validated for lions. The protocols described in this thesis must therefore be viewed as pilot studies which can provide value and insight into methods and assays that could be used to validate lion-based assay in addition to providing useful information for this study per se.

3.2.1 Cohort selection

From the results presented in Chapter 2 it was established that it is not possible in the current study to conclusively confirm on an ante-mortem basis which lions are infected with *M. bovis* or experiencing active disease. Additionally, there are indications that the diagnostic tests used in the current study might be lacking in specificity and/or sensitivity. This lack of specificity seems to be more pronounced in the captive lion population that presumably has a higher risk of exposure to MTC species other than *M. bovis*. Despite these difficulties encountered with the ante-mortem diagnosis of *M. bovis* infection or disease an attempt was made to at least classify a subset of the lions as probably infected or probably uninfected. This was done to facilitate the investigative comparative study needed to initiate research and start gaining insights into how *M. bovis* might be affecting the immune/inflammatory, energy metabolic, and reproductive endocrine systems in lions. Guidance for classifying the lions was taken from diagnostic algorithms used for classifying human TB cases (see Chapter 2).

Keeping the diagnostic limitations in mind and in order to get a more complete picture it was decided to make use of two separate cohort selection strategies to investigate the different metabolic systems. The first strategy was to compare lions presumably exposed to *M. bovis* (i.e. free-roaming/wild KNP lions) with presumably unexposed (i.e. captive lions). The second strategy

was to make use of the above mentioned selection criteria (described in Chapter 2 section 2.5) in order to classify lions as having a high probability of being infected with *M. bovis* (n = 20) or as having a low probability of being *M. bovis* or MTC infected (n = 13). While the probably infected lions were comprised of only wild lions, the probably uninfected lions included both wild and captive lions, thereby lessening the effect of possible bias introduced by the first strategy of comparing wild lions to captive lions.

Furthermore, for the purpose of the immune/inflammatory system investigation, biological markers were also in some instances investigated or compared between additional cohorts governed by the separate diagnostic tests used and discussed in Chapter 2. These additional cohorts were respectively lions classified according to the results obtained from the intradermal tuberculin skin test (ITT), and according to the QFT gene expression assay (GEA). Small sample sizes of TB Stat-Pak positive and BAL *M. bovis* positive animals prevented the use of these diagnostic results in meaningful statistical analyses. Due to the relative small sample size of the probably infected and uninfected cohorts, further subdivision into age and gender groupings was not done.

3.2.2 Sampling and sample assays

Blood samples were obtained from free-roaming (KNP) and captive (NZG, Ukutula, and PVT) lions. The capture and sampling procedures were described in Chapter 2 section 2.2. Blood samples for the analyses of cortisol and CRP concentrations were collected in red top serum tubes and kept on ice until further processing the same day. These samples were allowed to clot and were then centrifuged for 10 min at 3000 rpm after which the serum was divided into approximately 500 µl aliquots and stored at -20 ºC until further analysis (described in section 3.2.2.2). The plasma supernatant obtained from the pre-treatment step of the QFT-GEA (see Chapter 2 section 2.2.5) was used for cytokine analyses. The following sections describe the specific biomarker analyses in more detail.3.2.2.1 Cytokine analyses

The frozen plasma samples obtained as part of the pre treatment step of the GEA diagnostic test (See Chapter 2 section 2.2.5) were analysed in the Division of Molecular Biology and Human Genetics, University of Stellenbosch Faculty of Health Sciences, with the assistance of Dr. S Parsons and Dr. N Chegou. For a preliminary investigation, the QFT-NIL (unstimulated blood) and QFT-TB (stimulated with ESAT-6, CFP-10, and TB7.7 antigen simulating peptides) of seven lions were analysed with the MILLIPLEX® MAP Human cytokine/chemokine magnetic bead panel and the MILLIPLEX® MAP Canine cytokine magnetic bead panel (Merck (Pty) Ltd, South Africa).

These seven lions consisted of three ITT negative lions and four ITT positive lions. Cytokines analysed with the human panel were EGF, GM-CSF, IP-10, MIP-1a, TNF-α, VEGF, TGF-α, Fractalkine, and MCP-3. Cytokines analysed with the canine panel were GM-CSF, IFN-γ, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, IP-10, KC-like, MCP-1, and TNF-α. In many instances no

results were obtained due to values being below the detection range of the assay. When enough data was obtained statistical comparisons could be done, otherwise the data was visually inspected. The data were inspected using different comparisons with these results summarised as follows:

Unstimulated blood (QFT-NIL): ITT positive vs. ITT negative

- Significantly greater concentrations of KC-like (unpaired t-test, p = 0.0374) and IL-10 (unpaired t-test, p = 0.0401) in ITT positive lions (canine panel).
- Lower concentrations of IP-10 (visual inspection, human and canine panel) and IFN-γ (visual inspection, canine panel).
- IP-10 concentrations approximately 10 fold greater as measured by canine panel compared to human panel.

Stimulated blood (QFT-TB): ITT positive vs. ITT negative

- Significantly greater concentrations of KC-like (unpaired t-test, p = 0.0259) in ITT positive lions (canine panel).
- Greater concentrations of VEGF (Visual inspection, human panel), IP-10 (visual inspection, canine panel), IL-8 (visual inspection, canine panel), and TNF-α (visual inspection, canine panel).

Unstimulated blood (QFT-NIL) vs. Stimulated blood (QFT-TB) for respectively ITT positive and negative lions.

- Borderline non-significant decrease of VEGF (paired t-test, p = 0.0576, human panel) in ITT positive stimulated blood.
- Decreased EGF (visual inspection, human panel) in ITT positive stimulated blood.
- Decreased IP-10 (visual inspection, canine panel) in ITT negative stimulated blood.
- No significant differences for cytokine concentrations of the canine panel between stimulated and unstimulated blood of ITT positive lions. Only IP-10 showed a concentration increase in stimulated blood while all other cytokines had similar or slightly reduced concentrations in stimulated blood compared to unstimulated blood.

Comparison of response (ΔQFT = QFT-TB – QFT-NIL) between ITT positive and ITT negative lions.

- None of the ΔQFT values differed significantly between ITT positive and negative lions.
- Upon visual inspection the majority of cytokines showed lower ΔQFT values in ITT positive lions. IP-10 (canine) and IFN-γ showed increased ΔQFT values in ITT positive lions with a more pronounced difference between ITT positive and negative lions observed for IP-10. The greatest proportional decreases (visual inspection) in ITT positive lions compared to ITT negative lions was seen for the ΔQFT values of IL-10 and IL-8.

Since this was a preliminary investigation with a very small sample set, cytokines that showed a possible trend within the different comparisons were selected for further study. Cytokines selected for further study were VEGF (human panel) KC-like, IP-10, IL-8, IL-10 and TNF-α (canine panel). Analyses were done in accordance with the manufacturers' guidelines.

The cytokines identified in the above mentioned preliminary study were assayed in plasma samples for both the QFT-NIL (unstimulated) and QFT-TB (stimulated) whole blood from a total of 69 lions. In some lions the assay reported an "out of range below" value for one or more cytokines. The assumption was made that these "out of range below" values were zero or close to zero and were therefore regarded as such. Using the data from the unstimulated and stimulated blood a background corrected cytokine (ΔQFT) data set was compiled (i.e. ΔQFT = QFT-TB minus QFT-NIL). The ΔQFT data were then used for further statistical analyses.

3.2.2.2 Cortisol and CRP:

Frozen serum samples were sent to the Laboratory of Clinical Pathology, Department of Companion Animal Clinical Studies, Faculty of Veterinary Sciences, University of Pretoria (Onderstepoort campus). Cortisol concentrations were assayed with a solid phase, competitive chemiluminescent enzyme immunoassay (IMMULITE[®] 1000 (Cortisol), Siemens, South Africa). CRP concentrations were analysed with an immunoturbidimetric assay for canine CRP (Randox Laboratories Limited, Crumlin, United Kingdom). None of these assays were validated for lion samples.

3.2.3 Statistical analysis

For statistical analyses and some visualisation of the immune and stress markers, GraphPad Prism Version 4 for Windows (GraphPad Software, Inc., CA, USA) was used. Either a two-tailed unpaired t-test with Welch's correction (Welch test) was used when variances differed significantly or a two-tailed unpaired Mann Whitney test (MW test) when variances were not significantly different. The Kruskal-Wallis one-way ANOVA test was used for simultaneous comparison of three or more categorical groupings. When receiver operator characteristic (ROC) analyses were done with the infection subset data, case values comprised probably *M. bovis* infected lions and control values comprised probably uninfected lions.

Multivariate statistical analyses were done with R using RStudio for Windows (version 0.99.482) (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, http://www.rstudio.com/). Principle component analyses (PCA) were done for firstly just the ΔQFT data (data not normalised) and secondly with the cortisol and CRP results included (data normalised by means of the "scale" function in R with the formula: normalised x = (x – column mean)/column standard deviation). The PCA results were plotted as score and correlation plots. The different categorical variables were respectively super imposed on the PCA plots. Scatterplot-matrices were also compiled for the different markers and in some instances distance based cluster analyses were done using all or a selection of the stress and inflammation markers.

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Packages used in R:

- "base" package – PCA and cluster analyses
- "ggplot" package – Plotting of PCA score and correlation plots
- "car" package – Scatterplot-matrix and spearman correlations

3.3 Results

3.3.1 Cortisol

Mean cortisol concentrations of captive lions in this study were lower than previously described in captive and wild female lions (Table 3.1). Serum cortisol concentrations differed significantly (one-way ANOVA, $p < 0.0001$) between animal locations (Figure 3.1, A). KNP lions had the highest cortisol concentrations followed by NZG lions and then by Ukutula lions. Cortisol concentrations of wild (*M. bovis* exposed) lions were significantly higher (MW test, $p < 0.0001$) than captive (*M. bovis* unexposed) lions (Figure 3.1, B). Similarly, the subset of probably *M. bovis* infected lions had significantly higher (MW test, $p < 0.0001$) cortisol concentrations than the uninfected subset (Figure 3.1, B).
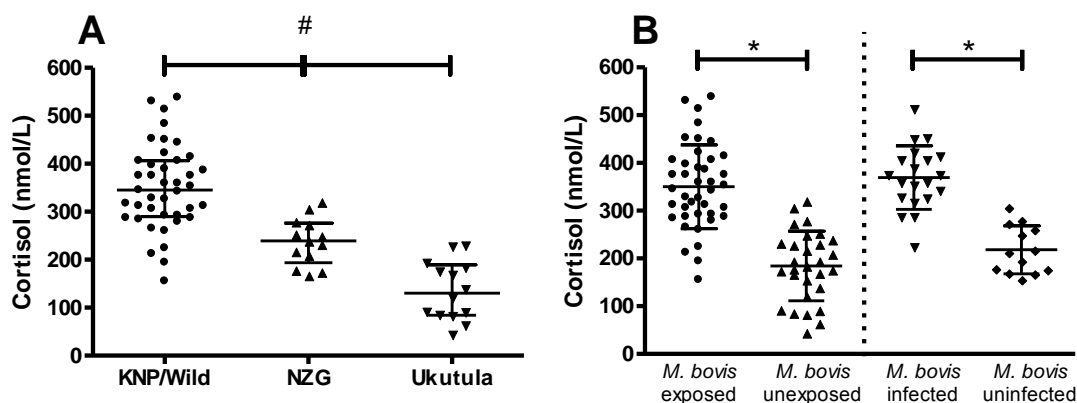
Figure 3.1: Scatter dot plots for cortisol concentrations with the data grouped according to sample location (A) and according to suspected *M. bovis* exposure and infection (B). Lines represent median and interquartile ranges. A – Cortisol concentrations differed significantly (one-way ANOVA, [#]$p < 0.0001$) between sampled lion populations. B – Cortisol concentrations were significantly greater (MW test, *$p < 0.0001$) in *M. bovis* exposed lions compared to unexposed lions (left of dotted line), and significantly greater (MW test, *$p < 0.0001$) in probably infected lions compared to probably uninfected lions (right of dotted line).

Wild/*M. bovis* exposed ITT positive lions had significantly higher (MW test, $p = 0.0418$) cortisol concentrations than wild ITT negative lions. The cortisol concentrations of the captive/*M. bovis* unexposed lions did not differ significantly between ITT positive and negative animals. Cortisol concentrations did not differ significantly (one-way ANOVA, $p = 0.6943$) between the gene expression assay (GEA) classification groups for wild lions.

Table 3.1: Summary of CRP and cortisol results for the different locations, exposure groupings, and infection subsets. Values given as Mean ± SD unless otherwise stipulated.

Biological marker	KNP/Wild/ *M. bovis* exposed	NZG	Ukutula	Captive/ *M. bovis* unexposed	Probably *M. bovis* infected	Probably *M. bovis* uninfected	Reference values*
CRP (mg/L)	373.9 ± 124.6	107.5 ± 47.09	211.1 ± 99.61	164.6 ± 91.92	372.6 ± 137.8	182.1 ± 148.2	160.1 ± 27.5, control cats, (Kajikawa et al. 1999) 242.9 ± 107.2, hospitalised cats, (Kajikawa et al. 1999)
Cortisol (nmol/L)	350 ± 87.97	238.2 ± 48.87	135.8 ± 61.31	184.3 ± 72.99	369.2 ± 66.54	218 ± 50.14	257.14 to 293.56 range of means, wild female lions, (Brown et al. 1993b) 264.86 mean, captive female lions, (Seal et al. 1976)

*Values as reported in the literature. When available, values for lions are given otherwise values reported for domestic cats are given. For studies conducted on different populations the values are given as a "Range of means" showing the lowest and highest mean concentrations reported.

3.3.2 CRP

The mean CRP concentrations) of *M. bovis* exposed and probably infected lions were higher than previously reported for hospitalised domestic cats (Table 3.1). Of the captive lions, Ukutula lions had significantly higher (Welch test, $p = 0.0026$) concentration of CRP than NZG lions but overall mean CRP concentration in captive lions was similar to that of healthy cats (Table 3.1). Lions classified as probably *M. bovis* uninfected had slightly higher CRP concentrations than healthy domestic cats (Table 3.1).

Serum CRP concentrations differed significantly (one-way ANOVA, $p < 0.0001$) between the different lion locations (Figure 3.2, A). Wild (*M. bovis* exposed) lions had significantly (Welch test, $p < 0.0001$) higher CRP concentrations than captive (*M. bovis* unexposed) lions (Figure 3.2, B). Inspection of the CRP concentrations for wild/*M. bovis* exposed lions showed two almost distinct aggregations within the data points (Figure 3.2). This could to a large part be explained by differences in CRP concentrations between the age classes. Juvenile wild/*M. bovis* exposed lions had significantly higher (MW test, $p = 0.0070$) CRP concentrations than wild adult lions. These age-related differences were not observed in the captive population or in the infection subsets.
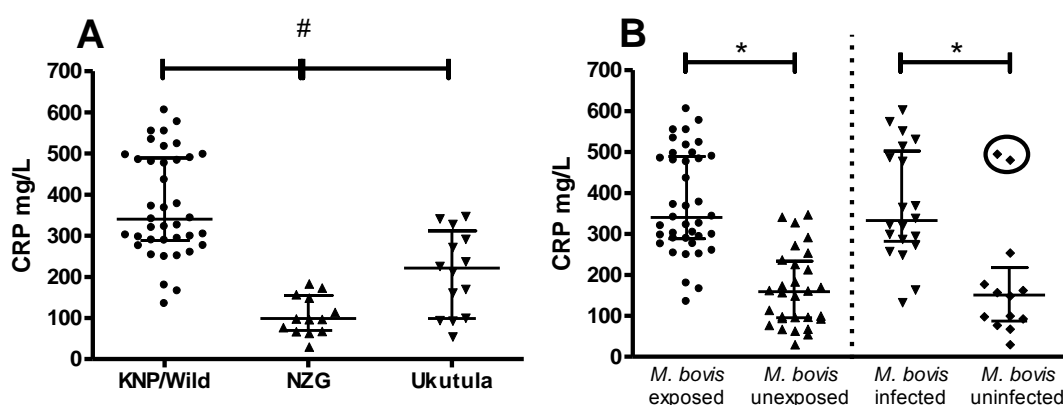
Figure 3.2: Scatter dot plots for CRP concentrations with the data grouped according to sample location (A) and according to suspected *M. bovis* exposure and infection (B). Lines represent median and interquartile ranges. A – CRP concentrations differed significantly (one-way ANOVA, #$p < 0.0001$) between sampled lion populations. B – CRP concentrations were significantly greater (MW test, *$p < 0.05$) in *M. bovis* exposed lions compared to unexposed lions (left of dotted line), and significantly greater (MW test, *$p < 0.05$) in probably infected lions compared to probably uninfected lions (right of dotted line).

The CRP concentrations in lions from the probably *M. bovis* infected subset were significantly higher (MW test, $p = 0.0007$) than that of the uninfected subset (Figure 3.2, B). The two outliers (encircled) were CRP concentrations from wild juvenile lions. By excluding

these outliers from the analyses, the differences between the infected and uninfected subsets were even more significant (Welch test, p < 0.0001).

The CRP concentrations in wild lions could not differentiate between ITT positive or negative lions or between the different GEA classifications.

3.3.3 Cytokines

A weakness of this study is that the performance of these assays has not yet been done. Therefore, the data is raw data as received. Note that it was not an objective to assess assay performance, rather this study is an initiative to assess which assay might yield results that can be used.

The ∆QFT data were visually inspected for outliers by means of Cleveland's Dot Plots and Box and whisker plots using the "base" package in R (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, http://www.rstudio.com/). Working with the assumption that disease will affect the concentrations of certain cytokines it is likely that diseased lions could have cytokine concentrations that were either much higher or lower than normal. To my knowledge there are no data available for the normal cytokine concentrations in lions. Therefore, when an outlier was identified it was further assessed in terms of whether the specific lion fell within the probably infected or uninfected subset. If a lion was included in these subsets the outlier associated with that lion was included in further statistical analyses. Outlier cytokine concentrations from lions not included in the subsets were excluded from further analyses.

A relative strong positive correlation was observed between the ∆QFT data of IL-8 and IL-10 (Figure 3.3).The histogram plots showed normal distribution for KC-like and IL-8. IP-10 data were skewed to the right and IL-10, TNF-α, and VEGF data were skewed to the left. Some changes are observed when looking only at the ∆QFT data of lions classified as probably infected and uninfected (Figure 3.4). The correlation between IL-8 and IL-10 was slightly weaker. The histogram plots showed that only IL-8 had normally distributed ∆QFT data. KC-like and IP-10 ∆QFT values were skewed to the right and IL-10, TNF-α, and VEGF were skewed to the left. There is a slight indication that increased ∆QFT values of KC-like are associated with an increase in VEGF and a decrease in IP-10.
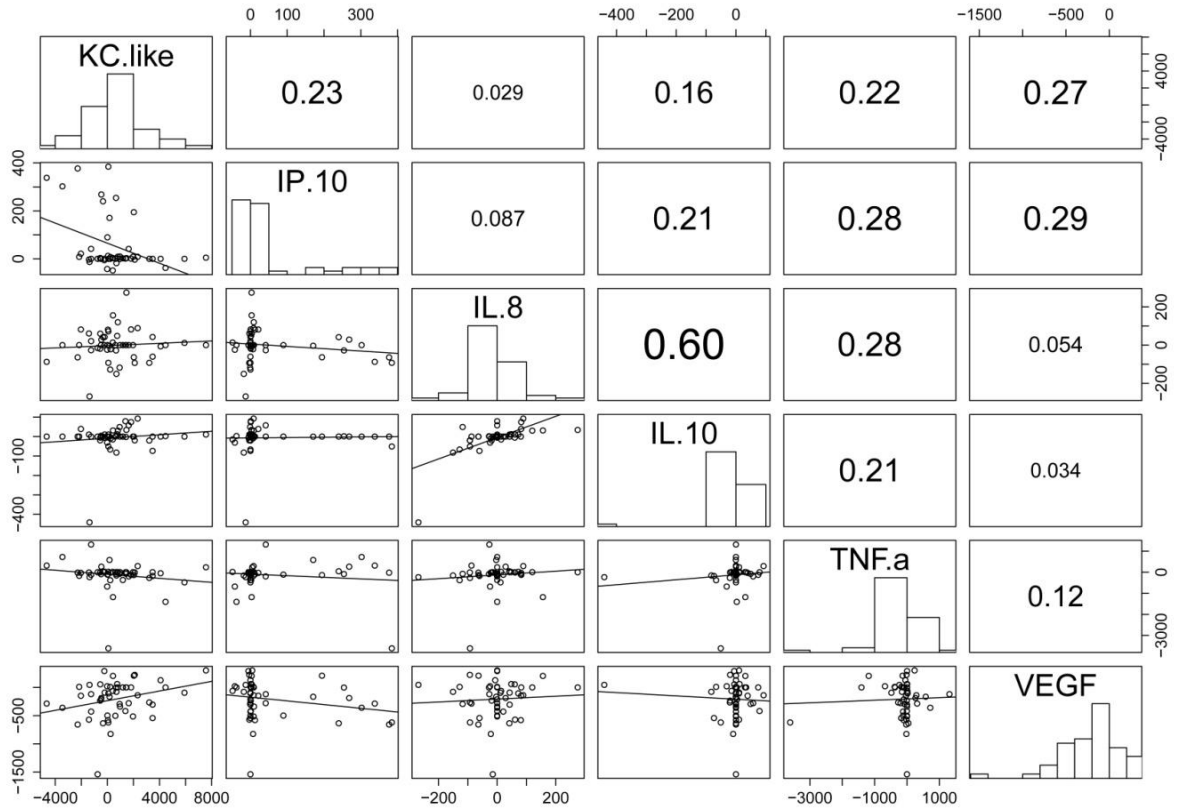
Figure 3.3: Scatterplot matrix of all the ΔQFT results with outliers excluded for the six analysed cytokines. Linear regression and Spearman's correlations between the different markers are indicated.
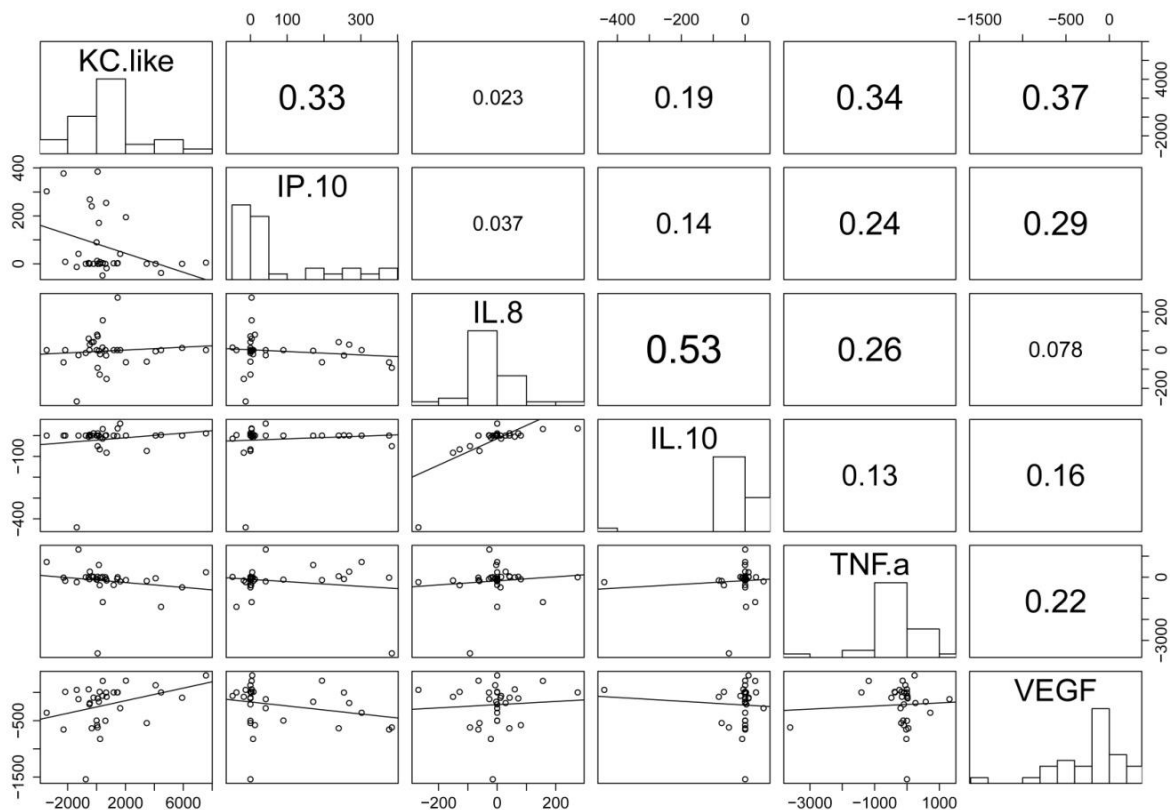
Figure 3.4: Scatterplot matrix of the ΔQFT results for the six analysed cytokines measured in the lions included in the probably infected and uninfected subsets. Linear regression and Spearman's correlations are between the different markers indicated.

Principal component analyses (PCA) were done on the ΔQFT data set of all the lions (excluding outliers) and the ΔQFT data set of the probably infected and uninfected subset lions. The summary of these results (Table 3.2) showed that for both data sets less than 60% of the variance could be explained by the first two principal components. Score and correlation plots were compiled for each of the data sets and different categorical variables were super imposed on it. In Figure 3.5 much overlap can be seen between *M. bovis* exposed and unexposed lions. However, it would seem that exposed lions were associated more with IP-10 and KC-like ΔQFT values while unexposed lions associated more with IL-8 and IL-10 ΔQFT values. Looking at the score and correlation plot of the subset ΔQFT data (Figure 3.6) much more overlap between infected and uninfected lions can be seen. By means of visual inspection the infected subset seems to be associated more with IP-10 and KC-like ΔQFT values with the uninfected subset only slightly associated more with IL-8 and IL-10 ΔQFT values.

Table 3.2: Summary of the PCA results for the first two principle components of each of the cytokine data sets. Values in bold indicate the cytokines that contribute the most to the relevant principle component.

| | All* | | Subset[1] | |
	PC1	PC2	PC1	PC2
Proportion of variance	0.30	0.25	0.30	0.26
KC-like	-0.40	**0.48**	-0.39	0.45
IP-10	0.40	-0.43	0.31	**-0.48**
IL-8	**-0.57**	-0.36	**-0.60**	-0.33
IL-10	**-0.51**	**-0.46**	**-0.55**	-0.45
TNF-α	-0.16	-0.21	-0.15	-0.07
VEGF	-0.26	**0.45**	-0.26	**0.49**

* PCA with ΔQFT cytokine concentrations of all the lions. Lions that had outlier cytokine concentrations and were not included in either the infected or uninfected subsets were excluded from the analyses.

[1]PCA with ΔQFT cytokine concentrations of only the lions included in the probably infected and uninfected subsets.
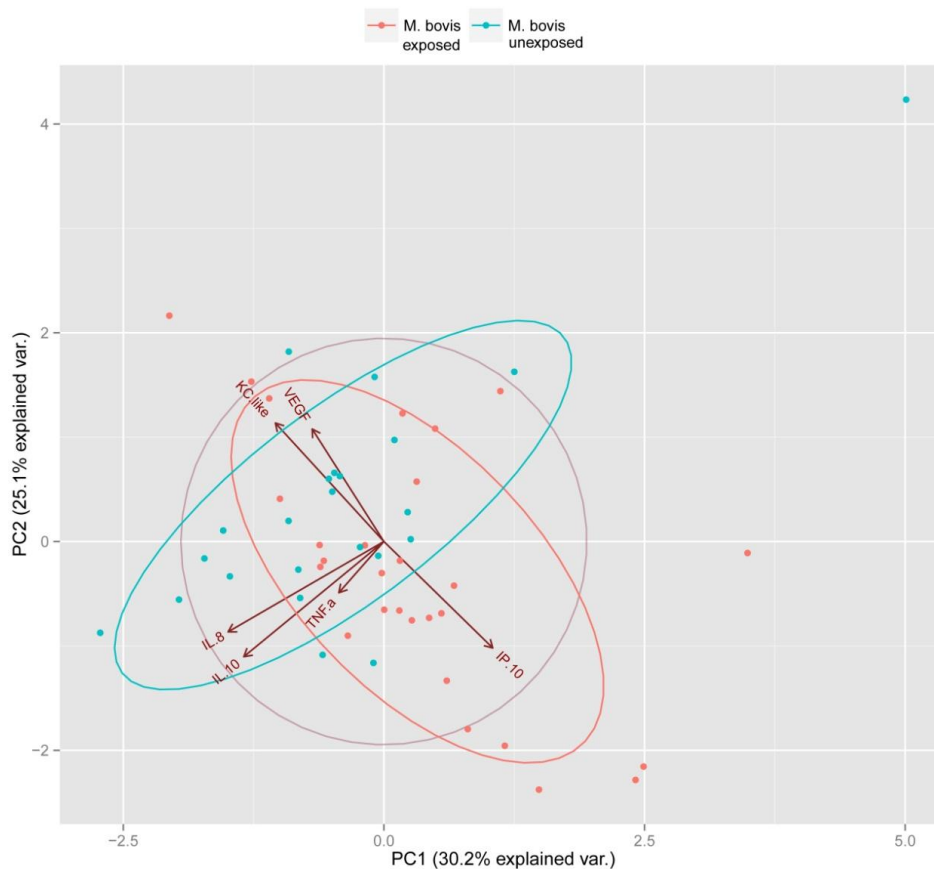
Figure 3.5: Score and correlation plot for all the ΔQFT cytokine data (outliers excluded). Different colours indicate different *M. bovis* exposure groups.
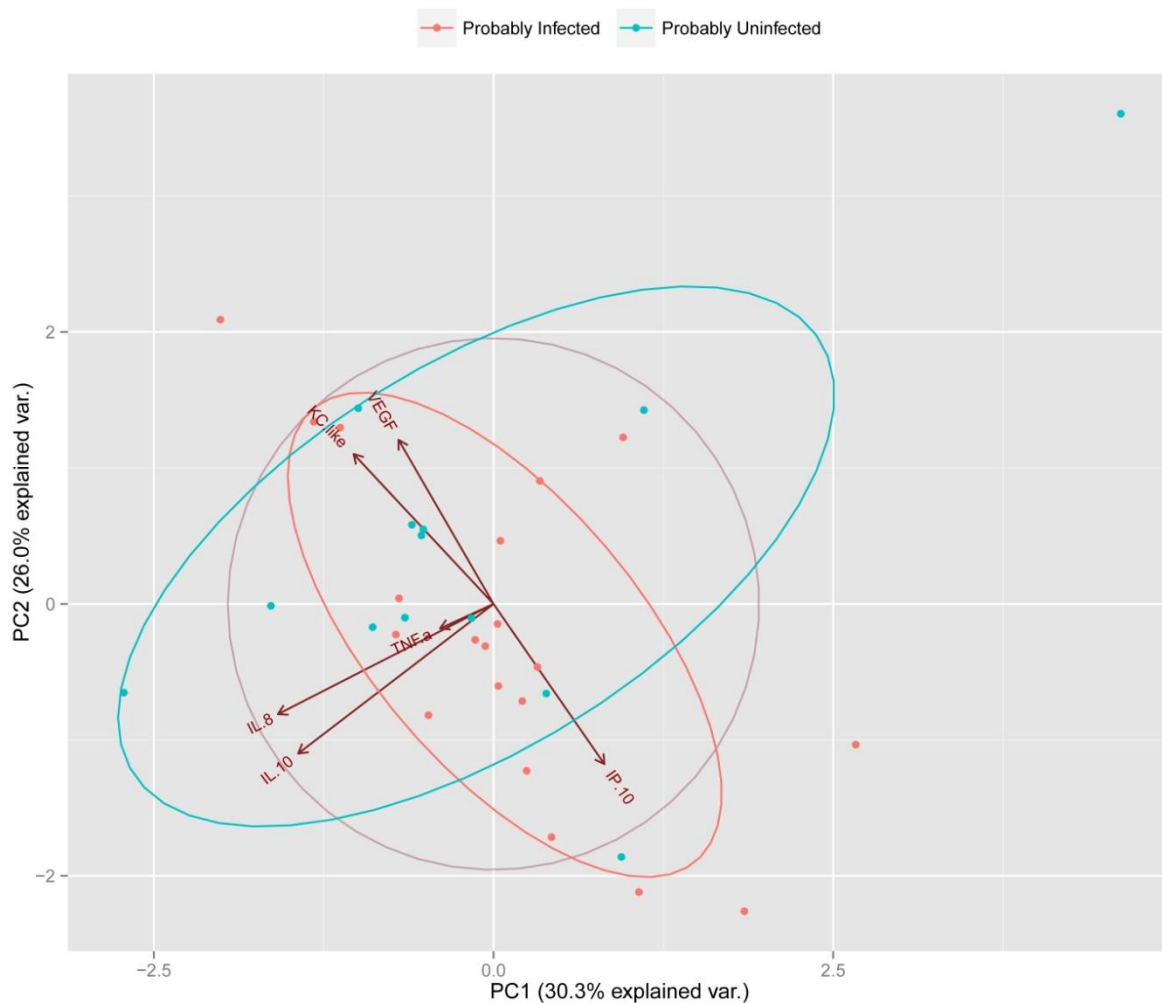
Figure 3.6: Score and correlation plot for the probably infected and uninfected subset lions' ΔQFT cytokine data. Different colours indicate different *M. bovis* infection groups.

Comparison of the ΔQFT (outliers excluded) showed significantly greater IP-10 values (Welch test, p=0.0005) and significantly lower VEGF values (Welch test, p=0.0104) in *M. bovis* exposed lions compared to unexposed lions. For the different infection subsets only IP-10 values were significantly greater (Welch test, p=0.0007) in the probably infected lions compared to the uninfected lions (Figure 3.8). The VEGF values did not differ significantly (Welch test, p=0.8824) between the infection subsets. However, three of the uninfected lions originated from the exposed area of which two had ΔQFT VEGF values that could be considered outliers in the uninfected subset. Excluding these three lions from the analyses resulted in infected lions having ΔQFT VEGF concentrations significantly lower (Welch test, p =0.0120) than uninfected lions.

A receiver operator characteristic (ROC) analyses were done with the IP-10 values of the infection subset lions (Figure 3.7). The highest specificity (75.00 %) and sensitivity

(92.31 %) to distinguish between probably infected and uninfected lions was at a cut-off of 3.3 pg/ml.
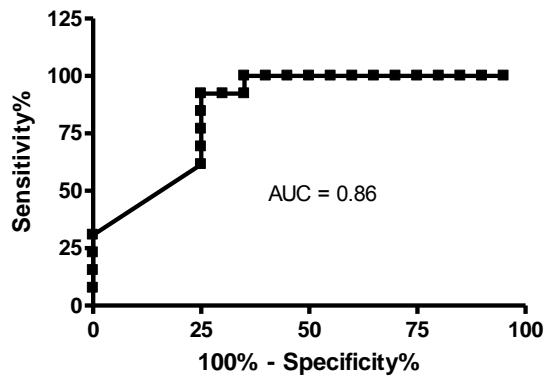
Figure 3.7: Receiver operator characteristic curve for IP-10 responses to antigen (ESAT-6, CFP-10, and TB7.7 antigen simulating peptides) stimulation. Case values comprised ∆QFT IP-10 concentrations of probably *M. bovis* infected lions. Control values comprised ∆QFT IP-10 concentrations of probably uninfected lions. AUC = Area under the curve.
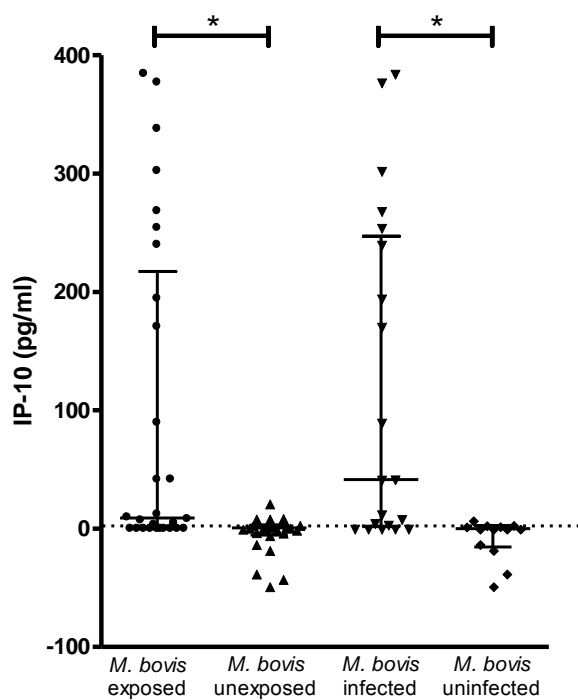
Figure 3.8: Scatter-dot plots of IP-10 concentrations for the different exposure end infection groups. Solid lines represent median and interquartile ranges. Dotted line indicates the optimal cut-off point for distinguishing between probably infected and uninfected lions. IP-10 concentrations were significantly greater (Welch test, *$p < 0.05$) in *M. bovis* exposed lions compared to unexposed lions, and significantly greater (Welch test, *$p < 0.05$) in probably infected lions compared to probably uninfected lions.

3.3.5 Multivariate analyses

Multivariate analyses were done with cortisol, CRP, and ΔQFT cytokine (outliers excluded) results included in the data set. For the purpose of this study these analyses were only done with results from the probably infected and uninfected subset of lions. As seen in section 3.3.2, CRP concentrations in wild adult lions differed significantly from juvenile lions. This, together with the small number of juvenile lions present in the uninfected subset lead me to do additional analyses with the results of the juvenile lions excluded.

In the infection subset data set, the strongest positive correlation was observed between cortisol and CRP (Spearman's, r = 0.56) (Figure 3.9). Slightly weaker positive correlations were observed between the ΔQFT values of IL-8 and IL-10 (Spearman's, r = 0.53), and between CRP and IP-10 (Spearman's, r = 0.54). When the juvenile lions were excluded from the infection subset data set (Figure 3.10) the above mentioned correlations were slightly stronger. Additionally, a slightly stronger positive correlation can also be seen between cortisol and IP-10 and a slightly negative correlation between cortisol and VEGF.
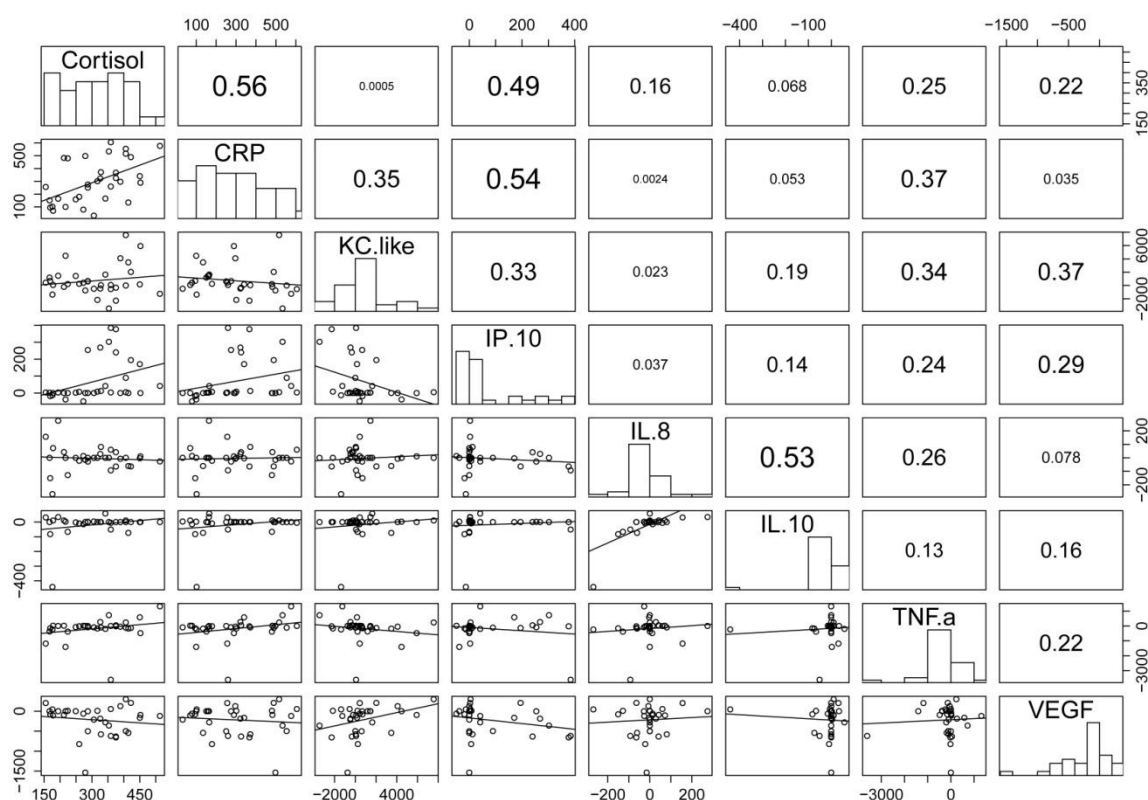
Figure 3.9: Scatterplot matrix with CRP, cortisol, and all the measured cytokines data of the lions classified as probably infected and uninfected. Linear regression and Spearman's correlations between the different markers are indicated.
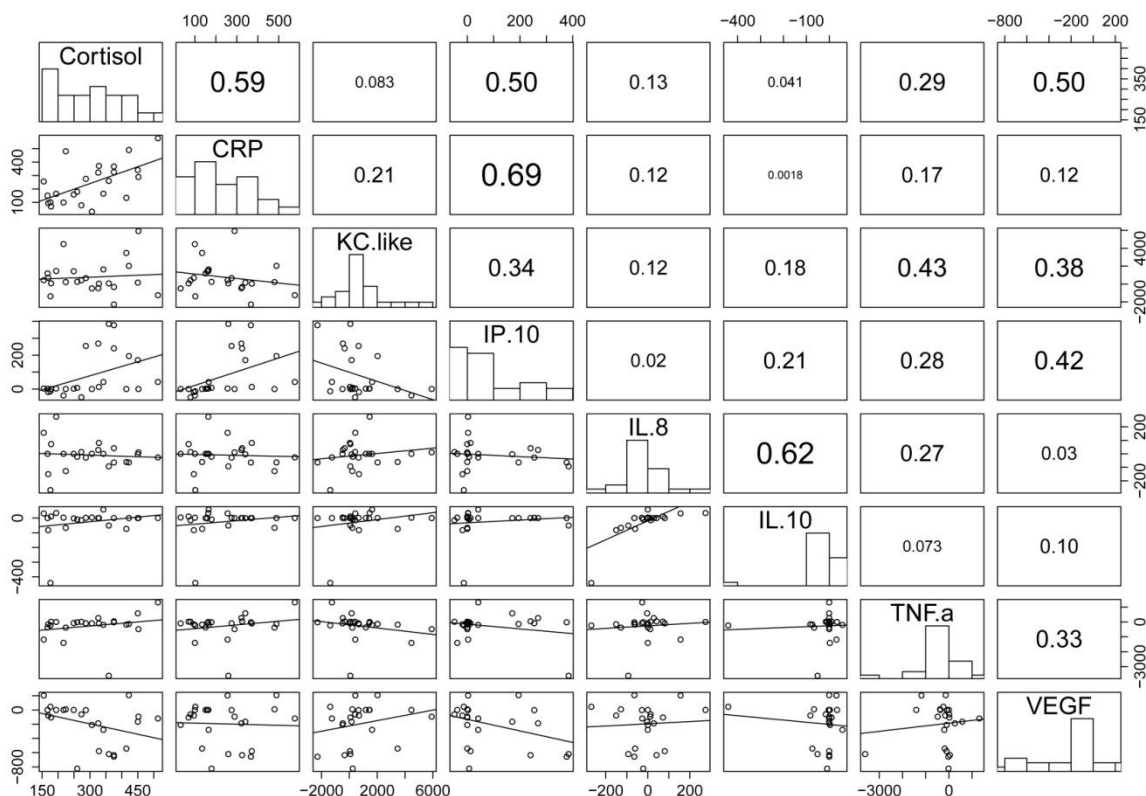
Figure 3.10: Scatterplot matrix with CRP, cortisol, and all the measured cytokines results of the probably infected and uninfected subset lions with data from juvenile lions excluded. Linear regression and Spearman's correlations are between the different markers indicated.

Principle component analyses were done with the stress/inflammation markers of the subset of lions classified as probably infected and uninfected as well as with the juvenile lions excluded from this subset. A summary of the PCA results for these two data sets can be seen in Table 3.3 with score and correlation plots generated with the PCA results in Figure 3.11 (probably infected and uninfected subset) and Figure 3.12 (infection subset excluding juvenile lions). In both instances the first two principle components (PC1 and PC2) only explains less than 50% of the variance within the data sets. For the infection subset cortisol and CRP contribute the most to the first component (PC1-1) while IL-8 and IL-10 contributes the most to the second component (PC2-1). With juvenile lions excluded cortisol, CRP, and IP-10 contribute the most to the first component (PC1-2) and the contribution of IL-10 and IL-8 is increased in the second component (PC2-2) when compared to the infection subset PCA results.

Exclusion of juvenile lions result in a clearer differentiation between probably infected and uninfected lions (Figure 3.12) compared to a greater overlap seen when juvenile lions are included in the analyses (Figure 3.11). In both instances the probably infected lions

associate more with increased values of cortisol, CRP and IP-10 while the uninfected lions associate more with increased VEGF and KC-like values.

Table 3.3: Summary of the PCA results of the stress/inflammation markers for all the lions classified as probably infected and uninfected ("Subset") and data of juvenile lions excluded ("Subset excl juveniles"). Values in bold indicate the biological markers that contribute the most to the relevant principle component. (PC1-1 and PC2-1 = Principle components one and two of the biological markers for all the probably infected and uninfected lions; PC1-2 and PC2-2 = Principle components one and two for the biological markers with data from the juvenile lions excluded)

	Subset		Subset excl juveniles	
	PC1-1	**PC2-1**	**PC1-2**	**PC2-2**
Proportion of variance	0.26	0.23	0.29	0.23
Cortisol	**-0.51**	0.02	**-0.52**	0.11
CRP	**-0.53**	0.03	**-0.50**	0.05
KC-like	0.18	-0.42	0.24	0.35
IP-10	-0.39	0.35	**-0.51**	-0.14
IL-8	-0.17	**-0.58**	0.04	**0.64**
IL-10	-0.36	**-0.51**	-0.18	**0.64**
TNF-α	-0.24	-0.13	-0.09	0.12
VEGF	0.25	-0.30	0.35	0.10
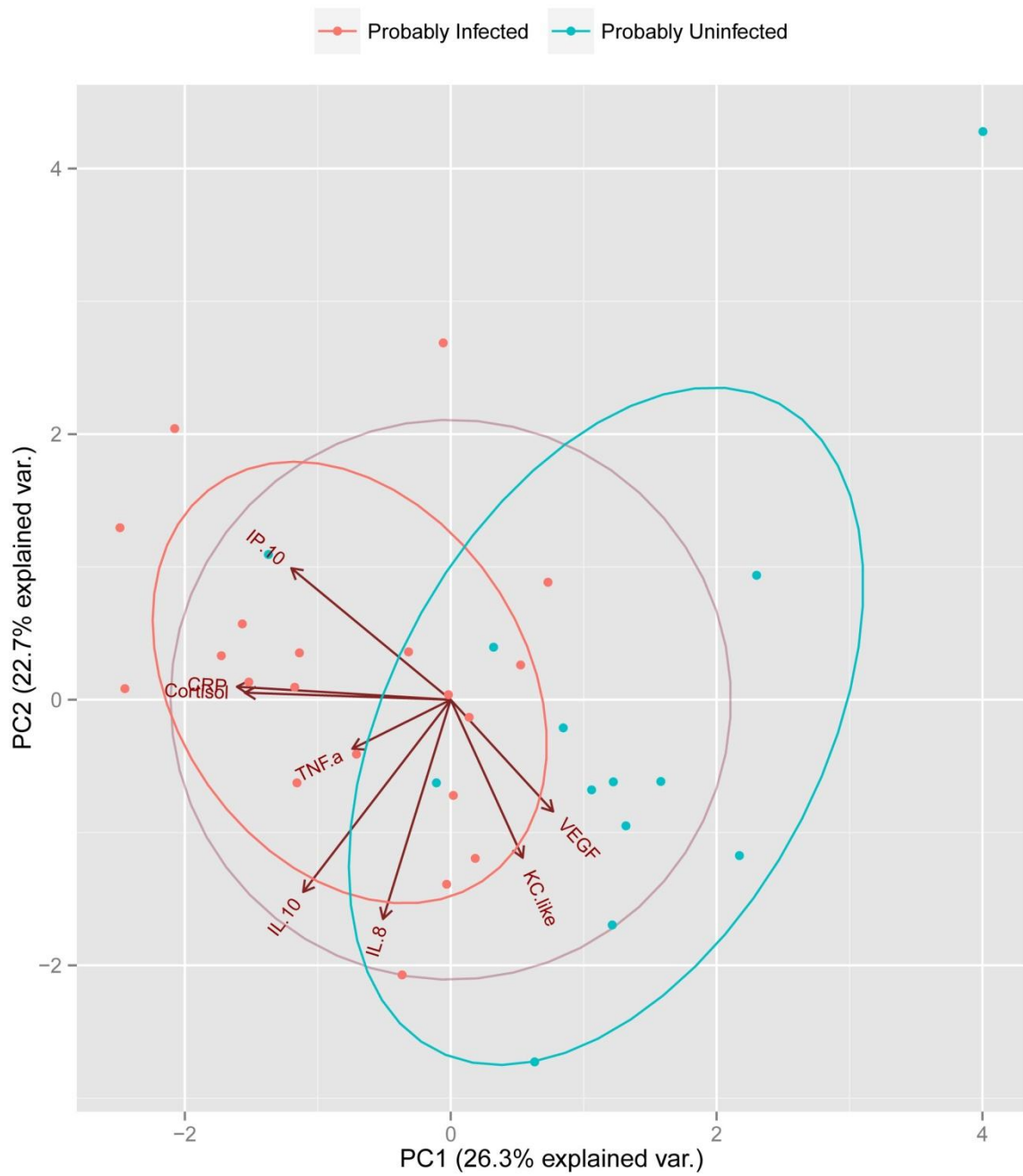
Figure 3.11: Score and correlation plot for PCA done with the subset of lions classified as probably infected and uninfected. Different colours indicate different *M. bovis* infection groups.
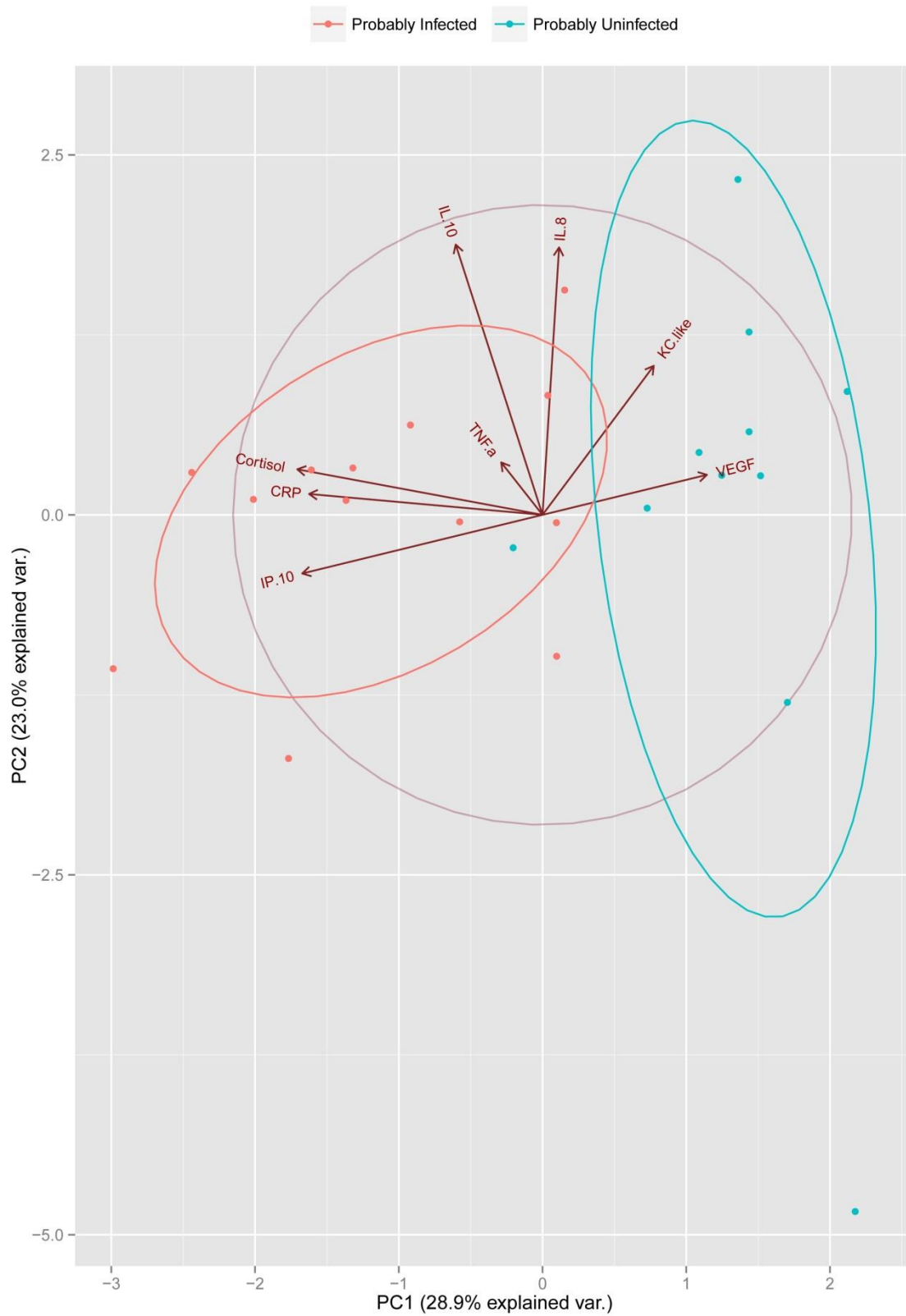
Figure 3.12: Score and correlation plot for PCA done with the probably infected and uninfected subset dataset excluding juvenile lions. Different colours indicate different *M. bovis* infection groups.

Distance based cluster analyses were done with the infection subset data that included all the stress/inflammation markers (Figure 3.13). No clear groupings were observed with various aggregations of probably infected and uninfected lions. Based on results obtained in the previous sections of this chapter a distance cluster analysis was done using only CRP, cortisol, and ΔQFT IP-10 data (Figure 3.14). Except for four animals (all originating from the KNP) that grouped incorrectly, clear aggregations of the probably infected or uninfected lions can be seen. With juvenile lions excluded (Figure 3.15) the split between infected and uninfected was even more pronounced, however, two infected lions were still grouped with the uninfected lions.
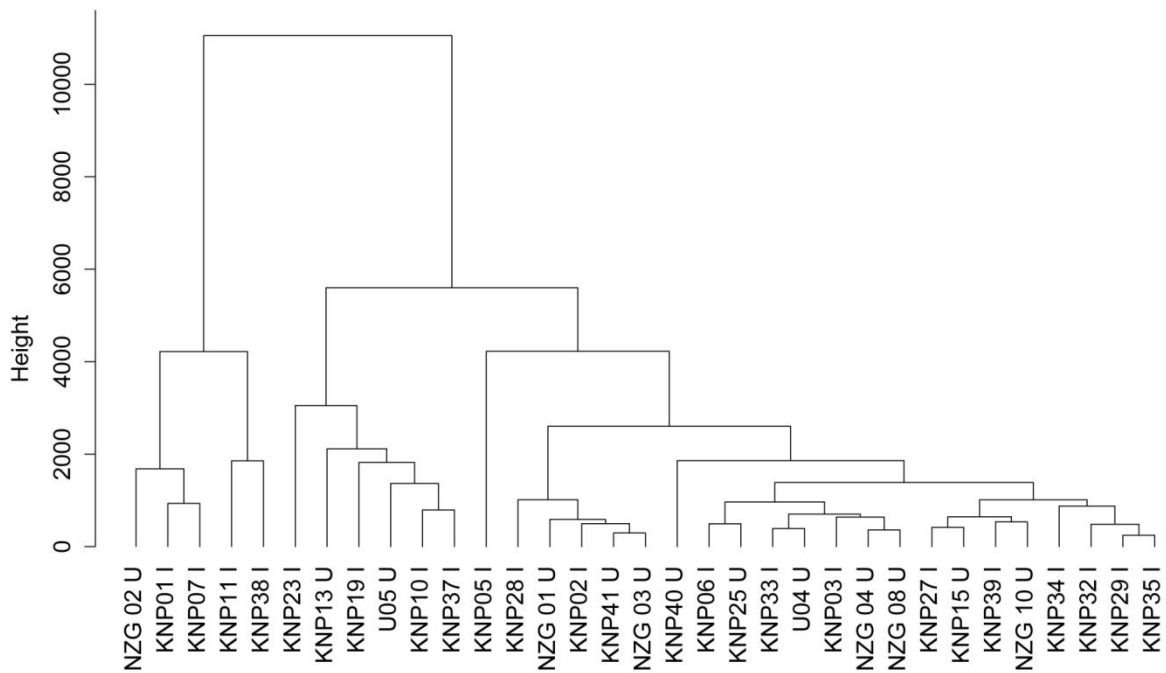
Figure 3.13: Cluster analyses dendrogram compiled with the probably infected and uninfected lion subset dataset. (I = probably infected, U = probably uninfected)
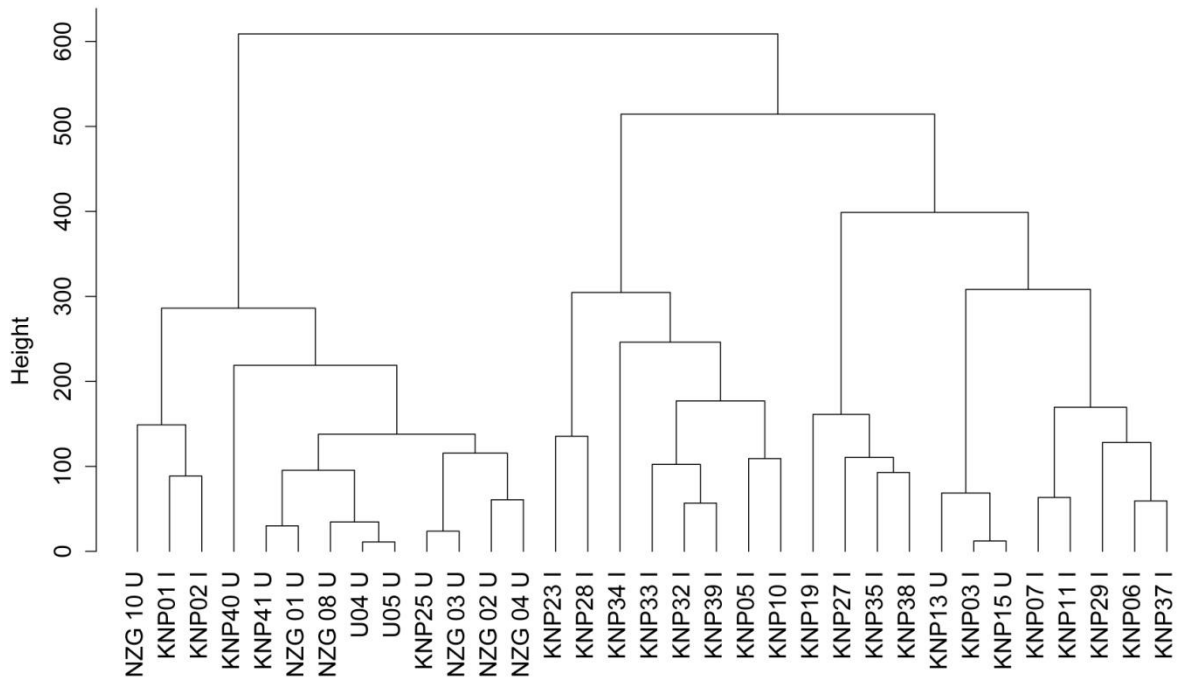
Figure 3.14: Cluster analyses dendrogram compiled using only cortisol, CRP and ΔQFT IP-10 concentrations from the probably infected and uninfected subset lions. (I = probably infected, U = probably uninfected)
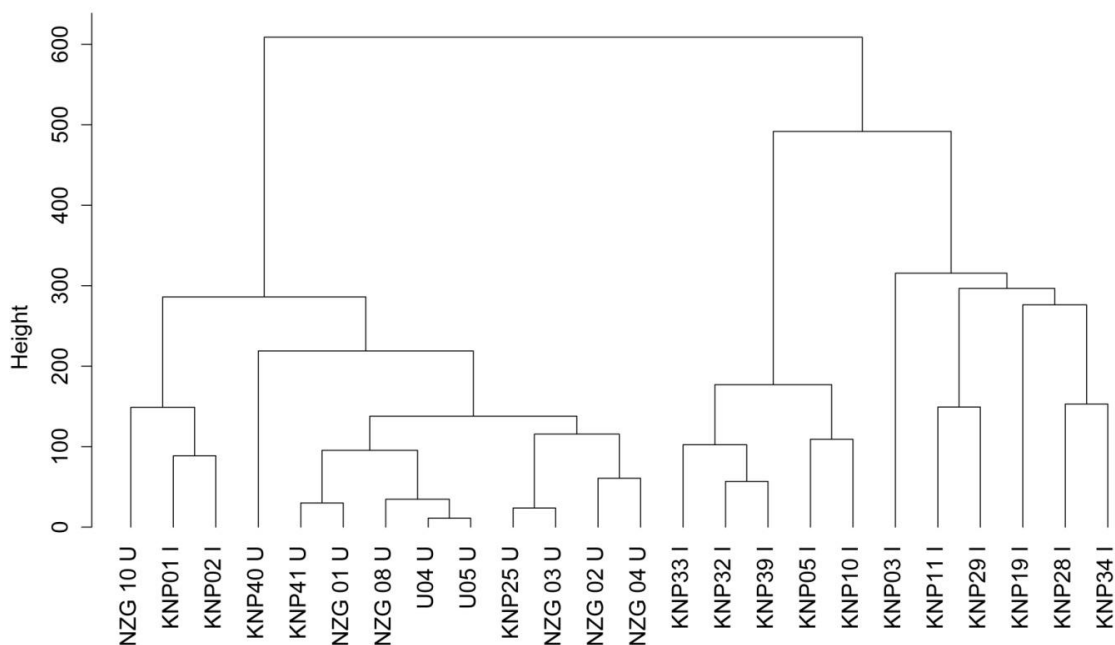
Figure 3.15: Cluster analyses dendrogram compiled using only cortisol, CRP and ΔQFT IP-10 concentrations from the probably infected and uninfected subset lions, excluding that of juvenile lions. (I = probably infected, U = probably uninfected)

3.4 Discussion

In order to better understand how a disease such as tuberculosis due to *M. bovis* affects a host animal such as lion, the ideal would be to experimentally infect and follow the progress of disease in lions under controlled circumstances. Unfortunately, we were not able to do this in the current study for a variety of reasons, which include that lions are on the CITES list and ethical permission for such a study would not have been granted at this time. However, with the lions and sample opportunities available to this study some insights can be gained that can hopefully serve as a foundation from which to do a proper experimental infection and disease study on lions some time in the future.

The main aim of this chapter is to gain insights and better understand the immune/inflammatory response of lions to *M. bovis* infection or disease. In order to do this, biological markers were compared between different lion populations that were regarded to be exposed or not exposed to *M. bovis*. From these two exposure groups, using the criteria described in Chapter 2 Section 2.5, some lions were grouped into either a probably infected subset or a probably uninfected subset. Although these subset classifications were subjected to strict selection criteria, none of these subset classifications were confirmed by means of necropsy. Therefore, although likely to be true with lions henceforth referred to as infected or uninfected, these subsets are not finally confirmed.

One lion (KNP05) was confirmed to be *M. bovis* positive by means of bacterial culture techniques. Where possible, the specific results obtained for this lion will be discussed.

3.4.1 Cortisol and CRP

At first glance the cortisol and CRP results represented in Table 3.1 and Figures 3.1 and 3.2 would suggest that lions exposed to or infected with *M. bovis* are experiencing higher levels of stress and inflammation than unexposed or uninfected lions. However, upon closer inspection and considering some demographic aspects this is not as straight-forward as initially thought.

3.4.1.1 Cortisol

Previously reported cortisol concentrations in unrelated captive and wild lion populations were in the same range (Table 3.1). Although these are only two reports one might, while considering the extensive differences between the two populations in those reports, expect that similar cortisol concentrations should be present in both the captive and wild populations from the current study. However, not only were the cortisol concentrations of captive lions in the current study lower than that of the wild (KNP) lions, it was also lower than the previously reported concentrations for both wild and captive lions. Additionally, the cortisol concentrations in wild lions from the current study were greater than previously

reported (Table 3.1). A possible reason for these differences of results from the current study compared to the previous reports could be the differences associated with different environmental factors and management practices between the studied populations, present and past. However, the fact that the cortisol concentrations were similar between a captive population and wild population on separate continents (wild = Africa, captive = North America) and many years apart brings this as a sole explanation into question. Without dismissing the possibility that differences in housing and management practices might have contributed to the observed differences between the cortisol concentrations and that of previous reports, a possible contributing factor could be exposure of the current wild/KNP lions to *M. bovis*, however, Brown et al. (1993b) did not mention or test for *M. bovis* in their study, limiting the ability to draw definitive conclusions. Results for the current study showed that captive *M. bovis* unexposed lions had lower cortisol concentrations than wild *M. bovis* exposed lions (Figure 3.1). (Note: it should be borne in mind that chronic stress can depress cortisol levels). However, because exposure does not necessarily indicate disease it might be worthwhile to further discuss other factors that might play a role, such as environmental factors mentioned earlier.

Firstly the observation that within the unexposed lions, cortisol concentrations differed between the Ukutula population and the NZG population might indicate that the housing environment or life history of the lions might be of importance. The majority, if not all, of the Ukutula lions were hand-reared and come into close proximity to humans very often. On the other hand the majority of NZG lions were not hand reared and at a relative young age are housed in enclosures that maintain a greater physical distance between humans and animals. Additionally, interactions between lions and humans occur more frequently and to a later stage of life in the Ukutula lions compared to the NZG lions. The cortisol differences between Ukutula and NZG lions could therefore possibly be ascribed to a greater stress response in the NZG lions due to the sedation and handling procedures. In line with this, the higher cortisol concentrations in KNP/*M. bovis* exposed lions could be ascribed to a greater stress response to sedation and handling as it was likely a completely novel experience for these lions. Alternatively, the fact that the wild lions were darted while around a carcass could also have contributed to the higher cortisol concentrations, since fighting for a spot around the carcass could possibly be more stressful. If we consider the assumption that captive lions might experience less stress during sedation and handling due to habituation to humans and handling events, it stands to reason that wild lions in their natural environment would be habituated to the regular occurrence of fighting over food as part of their normal behaviour and would therefore not experience feeding time as highly stressful. However, without being able to validate whether feeding around a carcass is an acute stressor or if habituation to fighting around a carcass lessens the possible stress

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effect, both of these points remain conjecture. It might also be possible that the differences between captive and wild lions could be ascribed to the circadian rhythm of cortisol production as can be seen in humans and rodents (Rook, 1999). All wild lions were sampled at night while all captive lions were sampled during the day. To my knowledge there is no data on circadian rhythms of cortisol production in lions, however, domestic cats do not seem to have circadian cortisol rhythms (Nogueira & Silva, 1997).

Since all the wild lions were FIV positive and only a small number of captive lions tested FIV positive it might be worthwhile looking at FIV as a possible contributor to the observed differences in cortisol concentrations between the wild and captive populations. A cross-sectional study that compared various immune-endocrine markers between two cohorts of domestic cats naturally infected with FIV for which one group received a six month treatment with antiretroviral medication found that the HPA axis of untreated cats was hyper-activated presumably due to chronic disease stress (Gómez et al. 2011). This included significantly higher levels of ACTH in untreated cats compared to treated cats and higher levels of cortisol, although not significant, in untreated cats (Gómez et al. 2011). They did not find a correlation between ACTH and cortisol and ascribed this to the fact that the adrenal glands are regulated by a variety of other factors and pathways that could alter adrenal sensitivity to ACTH and ACTH-independent stimuli (Gómez et al. 2011). On the other hand the report by Plechner (2004) mentioned that FIV positive domestic cats showed a trend of reduced cortisol together with high oestrogen and disturbed immune function. He questioned whether the presence of FIV lead to the reduced cortisol and immune imbalance or if the immune imbalance is a risk factor for the development of symptomatic disease in FIV infected cats (Plechner, 2004). These two reports are just examples of the uncertainty of how FIV might influence cortisol levels in domestic cats. Furthermore, the fact that FIV has species-specific strains or subtypes that interact differently with the respective hosts' immune system (Roelke et al. 2006) further complicates the ability to draw conclusion or make assumptions for lions from domestic cat studies. In the current study the highest cortisol concentration (249 nmol/L) of a captive FIV positive lion was only the 6th highest cortisol concentration in captive lions and lower than the lower quartile range of the wild lions' cortisol concentrations. This suggests that FIV is not a major driver of the differences in cortisol observed between captive and wild lions.

There is some evidence that the different cortisol concentrations between the exposed and unexposed lions could indeed be due to *M. bovis* infection or disease. Firstly, the fact that within the wild/exposed population, ITT positive lions had higher cortisol concentrations than ITT negative lions and secondly, some wild/*M. bovis* exposed lions were included in the uninfected subset and still cortisol concentrations differed significantly between infected and uninfected lions. Studies conducted on human TB patients identified

adrenal steroids as contributing factors in the regulation of host immunity against *M. tuberculosis* (Bini et al. 2015; Bottasso et al. 2010; Bottasso et al. 2013; Bozza et al. 2009; Bozza et al. 2007). In this regard it was established that contrary to the expected reduced cortisol levels in chronic stress situations, TB patients when compared to healthy controls, had increased cortisol concentrations (Bini et al. 2015; Bottasso et al. 2010; Bottasso et al. 2013; Bozza et al. 2007). While cortisol has anti-inflammatory characteristics the immune modulatory processes are complex and also involve other factors. Importantly, the interaction of the two adrenal steroids cortisol and DHEA (dehydroepiandrosterone) with the immune system was shown to influence the hosts' ability to fight infection/disease (Bottasso et al. 2010; Bozza et al. 2009; Bozza et al. 2007). In healthy controls, house hold contacts, and latently infected individuals a lower cortisol/DHEA ratio facilitated Th-1 response to mycobacterial stimulation and therefore a stronger protective CMI (Bozza et al. 2009; Bozza et al. 2007). In advanced TB patients an increased cortisol/DHEA ratio drove the immune response towards a Th-2 type response lessening the protective CMI response (Bottasso et al. 2010; Bozza et al. 2007).

The cortisol concentration of lion KNP05 was higher than the mean of the exposed lions but lower than the mean of the infected subset lions. Taking this together with the above into consideration the following likely conclusion might be drawn. The higher cortisol concentrations in wild or probably infected lions might be due to the presence of *M. bovis* infection or disease, although unable to conclusively prove this in the current study. Simultaneously, differences in handling stress might be present due to differences in habituation to human activities.

An alternative hypothesis might also be presented. Since cortisol responses are influenced by a variety of factors other than disease it might be that the increased cortisol has an inhibitory effect on the hosts' antimicrobial abilities (Brown et al. 1993a; Mahuad et al. 2004; Rook, 1999). Therefore, the innate higher cortisol concentration in wild lions is making them more susceptible to *M. bovis* infections/disease.

3.4.1.2 CRP

To my knowledge no previous data were available for CRP concentrations in lions. CRP concentrations of unexposed lions were similar to those of healthy cats (Kajikawa et al. 1999) while exposed and infected lions had CRP concentrations higher than that of hospitalised domestic cats (Kajikawa et al. 1999) (see Table 3.1). Interestingly, the uninfected lions had a slightly higher mean CRP concentration than the unexposed lions. This was still much lower than that of the infected lions and could possibly be ascribed to the relatively small sample size. Alternatively, since some of the supposedly uninfected animals

originated from the KNP it is possible that they were exposed but not infected, or were at a very early infection stage.

CRP is an acute phase protein produced in response to an acute inflammatory stimulus (Black et al. 2004). It is unlikely that the stress associated with the sedation and handling of the lions would affect the CRP levels in the blood. Therefore, the differences in CRP concentrations (Figure 3.2) observed between wild and captive lions cannot be explained by the different levels of habituation to handling by humans.

Closer inspection of the exposed and infected lions showed two, almost distinct aggregations of data points (Figure 3.2). Despite the fact that wild juvenile lions had higher CRP concentrations than adult lions, for the exposed and infected lions both juvenile and adult lions were represented in the two aggregations. This, together with the fact that no significant age related differences were observed in the unexposed lions and the infection subsets, might indicate that the age related differences observed in the exposed lions are possibly artefacts of another process. Although CRP is considered a marker of acute inflammatory responses it has also been used as a marker of systemic and or chronic inflammation (Bottasso et al. 2013; Dandona et al. 2004; Gan et al. 2004). Elevated CRP levels have been observed at TB diagnosis in humans, have been shown to decrease with TB treatment, and were correlated with disease severity and sputum bacillary load (Bottasso et al. 2013; Essone et al. 2014; Jayakumar et al. 2015). It can therefore be speculated that the aggregation of higher CRP concentrations in the exposed and infected lions come from lions that are experiencing advanced stages of TB. Unfortunately this cannot explain the two high CRP concentrations in the uninfected subset. Both of those CRP concentrations were from wild juvenile lions. This suggests that the selection criteria for the infection subsets may not have been strict enough, or that the *M. bovis* diagnostic tests are not sensitive enough, or that the aggregated higher CRP concentrations are due to inflammatory processes other than TB. More likely however, might be the presence of injury or a quite different infectious disease not controlled for in the current study. Additionally, the fact that the CRP concentration (259.44 mg/L) of KNP05 (a lion with active TB) was below the lower quartiles of the exposed and infected subsets further emphasizes that high CRP concentrations might not solely be due to *M. bovis* infection/disease.

3.4.2 Cytokines

A variety of cytokines take part in the regulation of immune/inflammatory responses in organisms. The scope of this study did not allow for the investigation of all known cytokine responses in light of a *M. bovis* infection. Rather, the main objective was to see if available human and canine assays had the ability to analyse lion cytokine concentrations while simultaneously aiding in the identification of cytokine responses specific to *M. bovis*

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infections in lions. A preliminary investigation (summary of results reported in section 3.2.2.1) identified six possible cytokines that could serve this role. The current study investigated the responses of TNF-α, IP-10, IL-8, IL-10, VEGF, and KC-like in whole blood (WB) stimulated with peptides simulating the *M. tuberculosis* complex (MTC) antigens ESAT-6, CFP-10, and TB7.7.

With part of this study aimed at furthering the knowledge of cytokine profiles in lions experiencing *M. bovis* infection or disease whilst also investigating the use of commercially available assays not specifically developed for lion samples, some data manipulation had to be done in an attempt to gain as much meaningful insights possible. One of the considerations for this was that in the current study it was not possible to definitively confirm disease, therefore, the selection criteria described in Chapter 2 were used to identify cohorts of lions that were considered to be probably infected with *M. bovis* as well as unlikely to be infected with *M. bovis* or any other MTC species. Since these two cohorts probably did not include animals with other MTC infections and since the probably uninfected cohort were comprised of both captive and wild lions, these two cohorts also served as a comparison base from which to describe or compare results obtained when looking at the lion populations at *M. bovis* exposure level (that could have been subject to bias resulting from geographical location). Keeping this in mind, lions not included in the probable infection cohorts, with cytokine concentrations considered to be outliers (in either unstimulated or stimulated blood), were excluded from further cytokine data analyses. This was done since the cytokines measured are not produced exclusively in response to *M. bovis* and could be produced in response to other infections or diseases. More specifically non-TB diseases or infections could affect cytokine levels in unstimulated blood whilst other MTC species could affect cytokine concentrations in MTC antigen stimulated blood. Furthermore, drawing from the methods described by Tebruegge et al. (2015) the cytokine data were background corrected by subtracting the cytokine concentrations of the unstimulated whole blood (WB) from that in the MTC antigen stimulated WB giving a ∆QFT cytokine response. The ∆QFT concentrations were then used for further analyses. Preliminary data exploration by means of scatter-plot matrixes and Spearman correlations (Figures 3.3 and 3.4), and principle component analyses (PCA) (Figures 3.5 and 3.6, and Table 3.2) were done. Only correlations with a Spearman's r > 0.5 will be discussed.

In both the exposure and infection groupings a relatively strong positive association was seen between IL-8 and IL-10 (Figures 3.3 and 3.4). This association became more apparent in the PCA's as IL-8 and IL-10 were the main contributors to the first principle component for both the exposure and the infection groupings (Table 3.2). Interestingly, the review by Couper et al. (2008) mentioned that IL-10 inhibits the production of IL-8 (See section 3.4.3 for further discussion).

While it was not possible to see clear groupings of exposed and unexposed lions, or infected and uninfected lions in the PCA score and correlation plots (Figures 3.5 and 3.6), these plots did show an association of the exposed and infected lions with increased IP-10 and decreased VEGF concentrations.

Of the six cytokines analysed, only IP-10 and VEGF showed significant differences when comparing exposed lions to unexposed lions. When cytokine responses were compared between the infected and uninfected subsets, only IP-10 differed significantly (Figure 3.8).

Previous studies reported the potential to use IP-10 in the diagnosis of human *M. tuberculosis* cases (Essone et al. 2014; Tebruegge et al. 2015). Additionally, the potential to use IP-10 as a sensitive biomarker in the diagnosis of *M. bovis* in African buffalo (*Syncerus caffer*) has also been shown (Goosen et al. 2015). IP-10 forms part of the Th-1-type immune response and is thought to play an important role in recruiting activated T-cells to sites of inflammation. The elevated IP-10 response in infected lions might therefore be an indication that these lions are experiencing a Th-1 type immune response. Interestingly, the IP-10 concentrations of the stimulated WB were approximately 100 to 1000 fold lower than that reported for human children (Lighter et al. 2009) and 10 to 100 fold lower than reported for African buffaloes (Goosen et al. 2015). Although the study by Lighter et al. (2009) made use of the same QuantiFERON®-TB Gold tubes to stimulate their WB, IP-10 analyses in their study was done with a human based multiplex system while the current study made use of a canine based multiplex system. Nevertheless, the IP-10 response in WB stimulated with *M. tuberculosis* antigens from the current study was able to discriminate between *M. bovis* infected and uninfected lions with 75% specificity and 92% sensitivity at a cut-off value of 3.3 pg/ml. This cut-off value is considerably lower than cut-off values proposed (1 486 pg/ml, or 4 557 pg/ml, or 2 155 pg/ml depending on the diagnostic assay test used) in a recent study for the diagnosis of *M. bovis* in African buffaloes (Goosen et al. 2015). However, Goosen et al. (2015) made use of an ELISA that targeted only IP-10 by means of an anti-bovine IP-10 capture antibody whereas the current study made use of a canine based multiplex system to measure lion IP-10. Further development of a lion specific IP-10 assay might therefore give a better yield that could facilitate a more robust cut-off point concentration.

With regards to VEGF concentrations, the current study showed a general trend of decreased VEGF concentrations in MTC antigen stimulated WB compared to the unstimulated WB. This decrease was significantly greater in exposed lions compared to unexposed lions but not for the infected lions compared to the uninfected lions. However, three of the lions included in the uninfected subset came from the exposed area. When their VEGF responses were excluded from the analyses the infected lions had a significantly decreased response compared to the uninfected lions. Alatas et al. (2004) and Matsuyama

et al. (2000) reported that serum VEGF concentrations in patients with active TB were higher than in healthy controls or patients that received disease treatment. Inspection of the VEGF concentrations of the unstimulated WB showed that exposed lions had a higher mean concentration than unexposed lions (data not shown). This was also the case for infected lions compared to uninfected lions with many of the uninfected lions having VEGF concentrations below the limit of detection of the assay. Considering this, together with the fact that macrophages have the potential to produce VEGF (Alatas et al. 2004; Matsuyama et al. 2000), might be an indication that the infected and exposed lions are experiencing macrophage activity greater than that of unexposed and uninfected lions. It can also be speculated that some form of VEGF inhibition occurs as part of the immune response against *M. bovis* in lions (infected lions), resulting in greater inhibition of VEGF than in lions for which the MTC antigens are novel. This inhibition of VEGF in stimulated blood might be similar to proposed inhibitory processes described for IP-10 in cattle blood stimulated with antigens ESAT-6/CFP-10, whereby activation of antigen-specific memory lymphocytes may be resulting in the production of inhibitory cytokines (Parsons et al. 2016). Alternatively, there might be some association between FIV infection and VEGF response to MTC antigen stimulation as all the exposed and *M. bovis* infected lions were FIV positive. This is further emphasised when the FIV positive lions were excluded from the uninfected subset resulting in more pronounced differences in VEGF responses between infected and uninfected lions.

Two of the pro-inflammatory cytokines in the current study (TNF-α and IL-10) have previously been assayed in lions believed to be infected with *M. bovis* (Maas, 2008). Maas (2008) made use of qPCR techniques to assay these cytokines, therefore, actual concentration values cannot be compared between this study and that of Maas (2008). However, trends in response to MTC antigen stimulation can still be compared between the two studies. Maas (2008) found that TNF-α responses in ITT+ lions were approximately two fold greater than in ITT- lions while differences between ITT+ and ITT- lions' IL-10 production was minimal. In the current study, TNF-α concentrations decreased after stimulation with MTC antigens. The mean decrease in exposed and infected lions was less than for unexposed and uninfected lions. Mean ∆QFT TNF-α concentration of exposed lions were 2.05 times higher than that of unexposed lions. Infected lions had mean ∆QFT TNF-α concentrations 2.25 times greater than uninfected lions. Despite these differences in mean concentrations the differences within the exposure and infection subsets were not statistically significant. TNF-α responses to antigen stimulation in the current study were thus similar to that described by Maas (2008). TNF-α is a pro-inflammatory cytokine associated with the Th-1 type immune response at the initial stages of infection and is inhibited by Th-2 type cytokines (Boddu-Jasmine et al. 2008; Thacker et al. 2007). One possibility might therefore be that a shift to a Th-2 type immune response might have already taken place,

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thereby inhibiting TNF-α production in antigen stimulated blood. However, this cannot explain the greater inhibition (although not significant) of TNF-α in the antigen stimulated blood of unexposed and uninfected lions compared to exposed and infected lions.

As with TNF-α, IL-10 responses in WB to MTC antigen stimulation also showed a general decrease in IL-10 concentrations after antigen stimulation. Mean ΔQFT IL-10 concentrations differed minimally between the exposed and unexposed lions, similar to the differences reported by Maas (2008). However, in the infection subsets mean ΔQFT IL-10 concentrations were 5.5 times greater in the infected lions compared to the uninfected lions. This was, however, not statistically significant. Despite this, the fact that the difference in ΔQFT IL-10 concentrations were more pronounced in the infection subset compared to the exposure groups might suggests an important role for IL-10 in *M. bovis* infections in lions and needs further investigation. Additionally, it should be noted that more of the exposed and infected lions failed to yield IL-10 results in either the unstimulated or antigen stimulated WB compared to the unexposed and uninfected lions. This might be in line with the findings of Thacker et al. (2007), who showed a decrease in IL-10 production as disease severity increased. However, the findings of Thacker et al. (2007) was the opposite to that of Boddu-Jasmine et al. (2008) in which IL-10 concentrations increased as disease progressed showing a gradual shift from a Th-1 type response to a Th-2 type response. Therefore, the lack of IL-10 in many of the infected lions might be an indication that the lions are still in a Th-1 type immune response with some infected lions converting to, or already experiencing a Th-2 type response. However, the reason for the general decrease of IL-10 in antigen stimulated WB compared to the unstimulated WB is unclear.

None of the other cytokines showed significant differences in MTC antigen stimulated ΔQFT responses between the different exposure or infection groups.

3.4.3 Multiple biomarker analyses

Due to the complexity associated with the immune/inflammatory response, some interactions, either direct or indirect, between the various biological markers used in this study might be present and should be tested for (Black et al. 2004; Brown et al. 1993a; Mahuad et al. 2004; Rook, 1999). This, if the interactions are in line with previous reports of such interactions, will serve the purpose of further elucidating the usefulness of the assays used as well as aid in describing immune/inflammatory response of lions to *M. bovis*. Additionally, it might draw attention to previously unknown interactions or differences in the immune response of lions to *M. bovis* compared to immune responses of other species. It might further be argued that the use of multiple biomarkers could eventually result in a definitive ante-mortem diagnostic. Because the main purpose of this study was to investigate the possible effects of *M. bovis* infections/disease on lions, multiple biomarker analyses was

limited to the infection subset data. Additionally, due to the age related differences observed in the CRP concentrations of wild lions, the multivariate analyses were conducted on the complete probably infected and uninfected lion data set, as well as the probably infected and uninfected lion data set with the juvenile lions excluded.

Exclusion of the juvenile lions led to more substantial correlations between the biological markers that showed either positive or negative associations in the scatter-plot matrixes (Figures 3.9 and 3.10). In addition to the relative strong positive association between IL-8 and IL-10 (as described in section 3.4.2), a stronger correlation was seen between CRP and IP-10 and a slightly weaker correlation between Cortisol and CRP. Additionally there were some indications that increased cortisol were associated with increased IP-10 but decreased VEGF.

IL-10 is an important immune-regulatory cytokine and has been reported to inhibit the production of many pro-inflammatory cytokines (Couper et al. 2008). IL-8, IP-10 and TNF-α are cytokines subjected to regulation by IL-10 (Couper et al. 2008). In the current study no correlation was observed between IL-10 and IP-10 or TNF-α whilst only IL-8 to some degree correlated positively with IL-10. These findings could suggest that the immune response to *M. bovis* is still Th-1 dominated in the lions studied, with IL-10 concentrations only starting to increase in order to drive the immune response to a Th-2 state. However, the chance that all of the lions are experiencing the same stage of infection or disease is highly unlikely. It might therefore be possible that the expected negative correlations were not observed as the sample comprises animals that vary in the type and stage of immune response.

Black et al. (2004) reported that CRP leads to increased production of TNF-α and IL-8, however, no positive correlations between CRP and TNF-α or CRP and IL-8 were observed in the current study. Smart & Casale (1994) reported that transendothelial neutrophil migration, that forms part of early phases of airway inflammation, is induced by TNF-α and is in turn IL-8 dependent. This is also to a large part reliant on TNF-α stimulated IL-8 production (Smart & Casale, 1994). However, the study by Friedland et al. (1992) showed that *in vitro* IL-8 production by human monocytes, after phagocytosis of *M. tuberculosis,* was independent of TNF-α. In the current study no correlations were observed between TNF-α and IL-8. Whether this is due to differences in lion immune responses compared to humans or due to failure of the assay is unclear. However, the latter might be more plausible.

When PCA's were done with all the biological markers, exclusion of the juvenile lions resulted in a clearer differentiation of infected and uninfected lions on the score and correlation PCA plot (Figures 3.11 and 3.12). From these PCA plots an association of infected lions could be seen with increased IP-10, cortisol and CRP concentrations further emphasizing the possible effects of a *M. bovis* infection on these markers. However, since

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all the probably infected lions were also FIV positive the possibility that other unknown co-infections are contributing to this association cannot be excluded.

Distance based cluster analyses were done with all the biological markers and visualised with a dendrogram (Figure 3.13). No clear groupings could be seen when all biological markers were included and resulted in various wild and captive lions being grouped together. For this reason a distance cluster analysis was done considering the results of the PCA's and the scatter-plot matrixes, using only the CRP, cortisol and IP-10 data (Figure 3.14). Except for four animals, two major groupings of infected and uninfected lions were obtained. The four lions that did not group correctly were two probably infected KNP lions that were grouped with an uninfected NZG lion and two uninfected KNP lions that grouped with a probably infected KNP lion. The only similarities of the two probably infected KNP lions with the uninfected NZG lion were that they were all adults. The two probably infected KNP lions were female and from the same pride but was completely removed from another probably infected juvenile male (KNP07) also from the same pride. The two probably uninfected KNP lions were juveniles from the same pride but of different genders while the probably infected KNP lion grouped with them was a male sub-adult lion from another pride. Removal of the juvenile lions from the cluster analyses did not affect how the two infected KNP lions mentioned above grouped. The remainder of the infected and uninfected lions were separated completely (Figure 3.15).The possibility, therefore, exists that those two probably infected KNP lions might have been classified incorrectly when the *M. bovis* diagnostic results were interpreted.

3.4.4 Known *M. bovis* infection (KNP05)

As was reported in Chapter 2, the only lion for which an *M. bovis* infection was confirmed by means of bacterial culture methods was KNP05. It was therefore of interest to see where this lion fits into all of the results.

As mentioned before, the cortisol concentration of this lion was lower than the mean cortisol concentration of the infected subset of lions but higher than the mean of the exposed lions. The CRP concentration was at the lower end of the scale (below lower quartile) of both the infected and exposed groupings. On the other hand, KNP05 had the highest ΔQFT IP-10 concentration of all the infected lions but not the exposed lions. The VEGF concentration in unstimulated WB was at the higher end of the scale of the infected lions. The ΔQFT VEGF concentration of this lion was similar to other infected lions that showed an above mean reduction in VEGF concentration after WB stimulation with MTC antigens. This lion also had a relatively high IL-10 concentration in its unstimulated WB compared to the other infected lions, for which many had immeasurably low IL-10 concentrations. This high IL-10

concentration is suggestive of a Th-2 type response, which is generally regarded as not protective against TB.

Therefore, were the diagnostic results not available, the cytokine results would be of more value to indicate possible *M. bovis* infection for this specific lion than would the CRP and/or cortisol concentrations. We could therefore conclude that cortisol and CRP are probably not good indicators of TB due to *M. bovis* while antigen specific IP-10 is. However, since this conclusion is based on only one animal, further evidence is needed to confirm this point.

3.5 Conclusion

At the beginning of this chapter (section 3.1) three objectives/questions were presented as guidance for the immune/inflammatory response investigation. Briefly, this study firstly confirmed that it is possible to obtain usable data of lion CRP and cortisol concentrations when using commercially available assays/laboratories. Secondly, it was possible to distinguish between the different cohorts of presumed *M. bovis* exposure pressures as well as between suspected infection categories using the data obtained for CRP, cortisol and some of the analysed cytokines. In order to address the third objective, namely to identify possible relationships between the different biological markers that could potentially indicate unexpected interactions, confirm usability of the assays, and/or give new insights into immune/inflammatory responses of lions to *M. bovis* that could aid the direction of future research, the following concluding remarks are offered:

The results in the current study indicate that TB in lions is connected to higher cortisol and CRP concentrations as well as variations in cytokine production different to that of unexposed or presumed uninfected lions. However, other unknown factors that are affecting the circulating concentrations of the biological markers in this study also seem to be at work. One of the major confounding factors of this study is that none of the presumed infection states could be confirmed with necropsy. Despite this, this study has identified some areas of potential importance for future research into the effects of *M. bovis* infection on lions with observations or suggestions as follows:

- There is a need for a proper controlled *M. bovis* infection experiment during which disease progression can be followed in association with the measurement of relevant biomarkers.
- There is a need for data on *M. bovis* infected lions that do not have any other immune compromising conditions such as FIV. This will have the additional benefit of shedding light on whether a FIV-*M. bovis* co-infection exacerbates the effect of *M. bovis* on lions, as *M. tuberculosis* does in humans with HIV.

- The cortisol and CRP results indicate that *M. bovis* infected lions are experiencing an inflammatory stress response. However, there are also indications that factors other than *M. bovis,* are contributing to higher cortisol and CRP concentrations in wild lions. This includes FIV infection (CRP), the novelty of physical restraint and sedation in wild lions (cortisol), other possible unknown infections or diseases in wild lions (cortisol and CRP), and a lack of knowledge about possible circadian rhythms in cortisol production. More research into the factors driving cortisol and CRP concentrations in wild lions is needed.

- Since cortisol levels can be influenced by a variety of factors other than disease the following alternative hypothesis for future study might be presented: "The innate higher cortisol concentrations in wild lions compared with captive lions might be contributing to an inhibitory effect on the antimicrobial abilities of the wild lions' immune response, thereby making them more susceptible to *M. bovis* infections/disease".

- A possible explanation for cytokine responses that do not conform to the expected responses might be due to localisation of cytokine production at the site of infection and are therefore not always measurable in whole blood samples. Analyses into cytokine production of lymphoid samples and/or tissues surrounding sites of pathology need to be related to cytokine profiles obtained from whole blood samples.

- In line with the above point, it is important when looking at cytokines to take into account the role of the cytokine in the immune system. This will then dictate if one should interpret the cytokine profile of unstimulated blood or tissues or that of the response to antigen stimulation. For example, as was seen in the current study, the VEGF response to antigen stimulation did not conform to what was expected while VEGF concentrations in unstimulated blood were more in line with what was previously seen in human *M. tuberculosis* patients.

- Cytokine assays need to be validated for lions or alternatively, analyses specific to cytokines in lions need to be developed. Results from this study suggest that suitable candidates to be focussed on in future studies might be IP-10 and VEGF. Further investigation into IL-8, IL-10 and TNF-α could also be useful.

- Whilst inhibition of certain cytokines in stimulated blood, such as has been seen in the current study might serve as an indication of possible interference from other substances with the assay, inhibitory processes of cytokine production and cytokine gene transcription from various mycobacterial species has been reported (Bai et al. 2011). Therefore, in line with the previous point of cytokine assay validation for lions, it will be necessary to establish if indeed the observed inhibition is due to mycobacterial or

immune response processes, or if it is due to interference from other unrelated substances.

- Despite the limited nature of the cytokine results, it does suggest that lions in the probably infected subset are indeed experiencing an immune response. IP-10 results indicate a possible Th-1 type response in the infected lions while IL-10 results indicate both Th-1 and Th-2 type responses (Increased IL-10 associated with Th-2; Low IL-10 concentrations associated with Th-1). TNF-α responses did not show a clear picture while VEGF responses were to some degree similar to that seen in early stage infections of *M. tuberculosis* in human patients.

- The scope of this study did not include the development of a diagnostic test for *M. bovis* nor for the development of a discriminating test of infection or disease stages. Despite the inability to definitively know the infection/disease status of the lions, it was possible with a relatively high sensitivity and specificity to distinguish between infected and uninfected lions when using the ∆QFT IP-10 results.

Much of the findings and conclusions were drawn from data gathered from lions of different ages and genders. In many cases, trends observed in the infection subsets, were present in the exposure groups but to a lesser extent. Considering this together with the fact that the infection subset classification was not confirmed with necropsy, the findings of this study serves as an indication that, when compared to the captive lions, the KNP lion population include lions experiencing either a Th-1 or a Th-2 immune response with subsequent elevated stress and inflammation. The presence of both Th-1 and Th-2 type responses is not unlikely since a population of lions exposed to *M. bovis* would include individuals at early asymptomatic stages of infection/disease (Th-1) and lions at later stages of disease (Th-2). At the moment it can only be speculated that this immune/inflammatory response is due to *M. bovis* infection/disease, although, the possibility of other causes cannot be excluded. Finally, these results are also an indication that the selection criteria proposed in Chapter 2 are to a large part sufficient in identifying lions probably infected with *M. bovis.*

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Chapter 4

Investigation into the possible effects of *M. bovis* on markers of energy metabolism in lions

4.1. Introduction

The ability of an animal to function and compete within the natural environment is to a large part reliant on the proper functioning and homeostasis of a magnitude of organs and metabolic pathways within the body. These bodily functions are interlinked and constantly interacting. For example, one of the functions affected by an immune/inflammatory response is energy homeostasis. However, energy homeostasis is not solely regulated by the immune/inflammatory response, and homeostasis can in turn have an effect on the immune/inflammatory response and the reproductive system (Blumenthal et al. 2009; Bottasso et al. 2010). The importance of energy metabolism in human TB has been established and can be seen in publications focussed on the links between diabetes and TB for example (Alladin et al. 2011; Broxmeyer, 2005; Gupta et al. 2011; Harries et al. 2009). Additionally publications investigating different aspects of the immune-endocrine-metabolic unit such as reviewed by Bottasso et al. (2010) and Blumenthal et al. (2009) further highlight this link.

It should therefore be possible to measure different biological markers in an organism that could help inform how the different *M. bovis* infection and disease states might be affecting the hosts' energy state. Additionally, this could also give insights into how other bodily systems are either affecting, or being affected by, the state of energy change associated with a mycobacterial infection.

One of the main non-specific clinical signs that lions are in a state of progressive active tuberculosis is emaciation (Viljoen et al. 2015). No information is available on the processes behind emaciation due to tuberculosis in lions nor are there any publications investigating energy metabolism of lions experiencing active tuberculosis. Some insights might be drawn from current knowledge on energy metabolism of healthy lions as well as from other mammalian models in order to better understand and describe the possible effects of tuberculosis on lion energy homeostasis.

Energy homeostasis, whereby the body's "fuel" stored in the form of adipose tissue is kept constant over long intervals, is maintained through a complex array of both short and long term regulatory mechanisms (Woods et al. 1998). These mechanisms, through positive and/or negative feedback loops, regulate when energy is stored or made available as well as how other bodily functions such as the immune and reproductive systems make use of the available energy (Bottasso et al. 2010; Woods et al. 1998). By looking at the compounds and hormones of these pathways one can start to gain insights into the energy state of an

organism. Included in this list of compounds and hormones are glucose, insulin, leptin, ghrelin, and by-products or biomarkers such as glycated haemoglobin, all of which will be discussed in more detail in the following sections.

Most of these hormones have also been confirmed to play roles in either or both the immune/inflammatory response and the reproductive system. Therefore, while this chapter will mainly focus on their roles in energy metabolism and homeostasis some reference will also be made to their alternative functions.

4.1.1 Glucose

Lions, like other felids, are obligate carnivores (Allen et al. 1995). However, as for other animals the main metabolic fuel for energy yield in carnivores such as lions is glucose (Behera et al. 2013). Levels of glucose in the blood depend on various factors, such as type of diet and the interactions of various hormones, including insulin, glucocorticoids, glucagon, thyroid hormones, pituitary and sex hormones, and epinephrine (Behera et al. 2013). The secretion of these hormones can vary between seasons and genders (Behera et al. 2013) The study by Behera et al. (2013) ascribed the seasonal and gender based variation of glucose concentrations in lion blood to the afore mentioned variation in factors able to affect glucose levels. Additionally, Behera et al. (2013) found that environmental temperature also affected glucose concentrations with these effects differing between males and females. They therefore suggested that these seasonal and gender based differences should be considered when using glucose (as well as other blood based markers investigated in their study) in disease diagnosis (Behera et al. 2013). In contrast, an earlier study found no significant differences in blood glucose concentrations between male and female lions (Jani et al. 2007). The Masters Degree dissertation of Erasmus (2008) found that blood glucose concentrations of captive lions differed significantly (ANOVA, $p = 0.004$) between age classes but not between genders or lion populations. Closer inspection of the data Erasmus presented showed that the lower and upper glucose reference range limits in cubs (< 1 year) were higher than that of juveniles, sub-adults or adults (Erasmus, 2008).

To my knowledge no data is available for glucose concentrations in lions in relation to tuberculosis. Yurt et al. (2012) did not find significant differences in glucose concentrations when comparing human TB patients with healthy controls or patients with other pulmonary disorders. However, when considering glucose levels together with insulin levels by means of the homeostasis model assessment of insulin resistance (HOMA-IR), human TB patients had significantly higher HOMA-IR values than any of the other study groups (Yurt et al. 2012).

4.1.2 Insulin

Insulin is a hormone produced in and secreted by the pancreas in response to circulating glucose concentrations. The main purpose of insulin is to enable cells to take up and utilise glucose or alternatively to store glucose as glycogen (Garrett & Grisham, 2005). In addition to the acute response of insulin to increased glucose concentrations, there are indications that insulin levels are influenced by overall fat stores (Backus et al. 2007; Woods et al. 1998) and also play a role in the synthesis of leptin (Woods et al. 1998). Interestingly, Backus et al. (2007) found that insulin levels in cats showed a stronger correlation with the amount of body fat than with dietary carbohydrate values. Both insulin and leptin, through communication with the central nervous system (CNS), have been shown to inhibit food intake and stimulate the loss of body fat (Woods et al. 1998). There are some indications that glucocorticoid hormones might serve as endogenous antagonists of insulin (and leptin) in the control of energy homeostasis (Woods et al. 1998).

In addition to insulin being a major factor in the regulation of cellular energy homeostasis, it is also hypothesised to play an important role in the regulation of phagocytosis by macrophages (Mao et al. 2011) as well as lymphocyte sensitisation and macrophage activation (Bell et al. 2007). The study by Bell et al. (2007) showed that an impaired insulin-glucose metabolism is present in human TB cases. Their results suggested that this impaired metabolism was to a large part due to a state of insulin resistance in TB patients (Bell et al. 2007). Whether this impairment is secondary to the infection or forms part of the process that makes an individual more prone to infection was still under debate in 2007 and more research was and is needed (Bell et al. 2007). The more recent study by Mao et al. (2011) hypothesised that that insulin resistance can serve as a marker and potential risk factor for active *Mycobacterium tuberculosis* infection.

To my knowledge no data is available on insulin concentrations in lions.

4.1.3 Leptin

Leptin is a single-chain proteohormone and is mainly produced by differentiated adipocytes (Meier & Gressner, 2004). However, production by other tissues such as the skeletal muscles, the liver, the fundus of the stomach, and placenta has also been described (Meier & Gressner, 2004). The primary role of leptin is to act on the central nervous system, in particular the hypothalamus, and suppress food intake while stimulating energy expenditure. In addition to its afferent role as a signal to the central nervous system of the body's fat status it has also been suggested to play a role in the hypothalamic-pituitary-gonadal (HPG) axis (Meier & Gressner, 2004) and the immune/inflammatory response (Bottasso et al. 2010; Yurt et al. 2012).

Under conditions of regular eating cycles, circulating leptin levels serve as an indicator of the proportion of adipose tissue. The synthesis of leptin is, however, modulated by several hormonal and non-hormonal factors. In humans and rodents leptin production is stimulated by over-feeding, insulin, and glucocorticoids. Fasting, cAMP, and β_3-adrenoreceptor agonists have been shown to suppress leptin production (Meier & Gressner, 2004). Additionally inflammatory mediators are also able to modulate leptin production (van Crevel et al. 2002) and prolonged inflammation is speculated to suppress leptin production (Yurt et al. 2012). Meier and Gressner (2004) mention a few aspects that need to be considered when interpreting measured leptin concentrations. Because body fat mass is the major confounding variable for leptin levels, leptin concentrations need to be referred to measures of percentage body fat such as the BMI. Additionally, serum leptin levels show a moderate circadian variation in humans, with a peak during the night. This variation could possibly be explained by the timing of meals and is probably due to the cumulative hyperinsulinemia that occurs during the course of a day (Meier & Gressner, 2004). Leptin also shows age dependent variance in children and adolescents. In adults, leptin levels were not affected by age but were gender dependent with higher concentrations in females than in males. Differences in soluble leptin receptors were also observed between genders (Meier & Gressner, 2004).

In sexually intact domestic cats, circulating leptin increased with increased fat and body mass (Backus et al. 2007). However, Backus et al. (2007) did not report increased leptin concentrations in gonadectomised cats within 17 weeks of the gonadectomy despite body mass increases of respectively 10% and 39% in males and females. This might be indicative of a link between the gonads and ability of leptin to indicate adipose energy abundance.

Leptin has also been shown to have direct effects on a series of peripheral tissues, suggesting a much more complex role of leptin in the body. Regulatory effects of leptin on the HPG axis and reproduction has also been identified (Meier & Gressner, 2004).

Further emphasis of this complex functioning of leptin in the body can be seen in the variety of leptin responses reported in the presence of *M. tuberculosis* infections. However, consensus has not been reached about the possible roles or effects of leptin in human TB. A decrease in circulating leptin concentrations was reported in human TB patients (Santucci et al. 2011; van Crevel et al. 2002). This is likely due to loss of body fat accompanying the disease (van Crevel et al. 2002). On the other hand, Yurt et al. (2012) did not find a significant correlation between BMI and leptin while Çakir et al. (1999) found that patients with pulmonary TB had higher leptin concentrations that further increased (although not statistically significant) during the course of treatment. Chronic stimulation of pro-inflammatory cytokines has been shown to decrease leptin levels (Santucci et al. 2011; van

Crevel et al. 2002) while hypoleptinemia was associated with an increased risk of infections (in general) (Santucci et al. 2014) or may contribute to increased TB disease severity (van Crevel et al. 2002). Some studies reported immunomodulatory properties for leptin (Santucci et al. 2014). In contrast, Yurt et al. (2012) suggested that leptin does not seem to play a role in host immunity against *M. tuberculosis* nor does it form part of the pro-inflammatory response in human pulmonary TB. Additionally, Santucci et al. (2014) showed that administration of leptin was not able to correct immune imbalances due to TB.

To my knowledge no data is available on leptin concentrations in lions.

4.1.4 Ghrelin

Ghrelin is predominantly produced by the epithelial cells lining the fundus of the stomach. The placenta, kidney, pituitary, and hypothalamus also produce small amounts (Meier & Gressner, 2004). Many of the effects of ghrelin are opposite to those of leptin. It has been established that ghrelin stimulates food intake in humans and rodents and plays an important role in energy homeostasis. It increases fat tissue by decreasing fat oxidation and has been shown to have metabolic, cardiovascular, antiproliferative, and orexigenic effects (Meier & Gressner, 2004).

In (human) adults, ghrelin levels can increase up to two-fold just before a meal and decrease to the lowest levels within an hour after eating. In individuals experiencing a negative energy balance, increased ghrelin concentrations were reported while obese humans had decreased ghrelin concentrations (Meier & Gressner, 2004). Studies done on obese or anorexic patients showed that after dietary intervention ghrelin levels increased in obese patients and decreased in patients with anorexia nervosa, suggesting ghrelin as a good marker of nutritional status (Meier & Gressner, 2004). Additionally, a possible link between ghrelin and glucose concentrations has also been suggested, whereby persistent low levels of ghrelin in diabetic children might suggest a defensive mechanism against hyperglycemia (Meier & Gressner, 2004). Similar to humans, plasma ghrelin levels of domestic cats decreased with increased body mass (Backus et al. 2007). Backus et al. (2007) also found that plasma ghrelin concentrations were inversely correlated with increased dietary fat concentrations. Additionally, they reported gender based differences in plasma ghrelin concentrations similar to those reported in humans, with females having greater concentrations of ghrelin than males (Backus et al. 2007).

To my knowledge, not much has been published on ghrelin in the context of *M. tuberculosis* infections. Increased levels of circulating ghrelin were observed in human TB patients from two separate studies (Santucci et al. 2011; Yurt et al. 2012). Yurt et al. (2012) concluded that the ghrelin concentrations observed in the context of the leptin

concentrations served as an indication that a catabolic/anabolic imbalance was present in TB patients.

4.1.5 Haemoglobin A1c (HbA1c)

To my knowledge no information is available with regards to HbA1c concentrations or functions in lions. To elucidate its possible value as a biological marker for energy homeostasis a brief overview of knowledge sourced from human studies will be given.

The most abundant minor haemoglobin in the human erythrocytes is HbA1c. It makes up approximately 4% of the total haemoglobin in adult human erythrocytes. HbA1c is formed by means of a non-enzymatic chemical condensation of haemoglobin and glucose. This is an extremely slow process occurring throughout the 120 day life-span of an erythrocyte (Bunn et al. 1976). This becomes important when one considers that blood glucose levels can vary considerably during the course of a day with a single sample unable to shed light on the general regulation of blood glucose concentrations in an individual. On the other hand, HbA1c (given as percentage of haemoglobin that is glycated) has been shown to indicate the average blood glucose spanning a five to 12 week period (Nathan et al. 2008). This provides better insights into the ability of an individual to regulate circulating glucose levels.

This link between blood glucose and HbA1c has led to the use of HbA1c as a biological marker to identify diabetes and prediabetes (Lyons & Basu, 2012). An approximate two-fold increase in HbA1c has been reported in patients with diabetes mellitus (Bunn et al. 1976). HbA1c has also used to monitor the adequacy of treatment in diabetic patients (Nathan et al. 2008).

Unfortunately, interpretation of HbA1c results has been shown to be not as straight forward as could be hoped. The study by Rohlfing et al. (2002) showed that the correlations between HbA1c and plasma glucose differed at different times of the day. This resulted in certain plasma glucose levels more accurately predicting HbA1c than others. For example, Rohlfing et al. (2002) found that fasting plasma glucose levels progressively underestimated HbA1c at increasing plasma glucose levels. This led Rohlfing et al. (2002) to suggest that caution should be used in only using fasting plasma glucose levels as a measure of long-term glycemia or as a surrogate measure of mean plasma glucose.

The average life span of an erythrocyte is 120 days, but erythrocytes of all ages contribute to the final HbA1c measurement (Rohlfing et al. 2002). In this regard Rohlfing et al. (2002) found that plasma glucose levels in the preceding 30 days, and therefore resultant HbA1c levels, contribute approximately 50% to the final result while plasma glucose levels from between 90 and 120 days before only contribute approximately 10%. Therefore, HbA1c could be considered a "weighted" average of blood glucose levels during the preceding 120

days (Rohlfing et al. 2002). This can then also become a possible confounding factor in the correct interpretation of HbA1c results since other conditions that reduce erythrocyte life span can also result in lowered HbA1c (Nathan et al. 2008).

In two separate studies, whilst taking most of the above into account, Nathan et al. (2008) and Rohlfing et al. (2002) presented linear regression equations from which to calculate the estimated average glucose (EAG) concentrations (Nathan et al. 2008) or the mean plasma glucose (MPG) concentrations (Rohlfing et al. 2002) from HbA1c values. However, the review by Lyons & Basu (2012) cautioned the use of HbA1c as a consistent measure of mean blood glucose since there is evidence that factors such as age, race, genetics and physiology are also biological determinants of the HbA1c-blood glucose relationship.

4.1.6 Aims

While keeping in mind that the biological markers discussed above will not give a complete picture of energy homeostasis in lions exposed to *M. bovis* and the possible resultant infection or disease effects, this part of the study will aim to investigate or address the following main points.

1) Is it possible to make use of commercially available assays or laboratories, not specifically designated for analyses of lion samples, to generate usable data for the biological markers of energy metabolism mentioned in the introduction of this chapter?

2) Will it be possible with the data generated to distinguish between lions of different exposure or presumed infection statuses?

3) Since, to my knowledge, none of these above mentioned markers have been investigated in lions in relation to *M. bovis* infection or disease, this study will initiate research into this aspect and where possible develop hypothesis or suggest directions for further research.

4) Will it be possible with the current study model to generate relevant knowledge to describe *M. bovis* infection or disease effects on lion energy metabolism?

4.2. Material and methods

It should be noted that there are no, or very few biological assays validated for lions. The protocols described in this thesis must therefore be viewed as pilot studies which can provide value and insight into methods and assays that could be used to validate lion-based assays in addition to providing useful information for this study per se. The biomarker assays chosen were selected while keeping cost effectiveness and ease of access in mind. Therefore, in some instances when feline assays were available but not easily accessible and cost was too high, human, or canine assays were selected instead.

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4.2.1 Cohort selection

For reasons described in Chapter 3 section 3.2.1 selection of the cohorts used for the comparative investigation were based on the likelihood of lions to be exposed to *M. bovis* (i.e. wild presumably *M. bovis* exposed vs. captive presumably unexposed lions) and secondly on the probability of being infected (i.e. probably *M. bovis* infected vs. probably uninfected lions).

In some instances, where relevant, the time since the last meal was also considered as this might directly influence the circulating concentrations of some of the markers. Additionally, in light of the findings described in Chapter 2, energy metabolism markers of wild/*M. bovis* exposed lions were inspected with regards to the diagnostic results obtained with the intradermal tuberculin skin test (ITT) and the QFT gene expression assay (GEA). Small sample sizes of TB Stat-Pak positive and BAL *M. bovis* positive animals prevented the use of these diagnostic results in meaningful statistical analyses. Due to the relative small sample size of the infected and uninfected subsets, further subdivisions into age and gender groupings could not always be done.

4.2.2 Sampling

Blood samples were obtained from free-roaming (KNP) and captive (NZG, Ukutula, and PVT) lions. The capture and sampling procedures were described in Chapter 2 section 2.2. Blood samples for the analyses of glucose, leptin, ghrelin, and insulin concentrations were collected in red top serum tubes and kept on ice until further processing the same day (in general not more than four hours passed between sampling and processing). These samples were allowed to clot and were then centrifuged for 10 min at 3000 rpm after which the serum was divided into approximately 500 µl aliquots and frozen at -20 ℃. In addition to the serum tube a blood sample was collected in an EDTA (lavender top) tube that was kept on ice and frozen at -20 ℃ on arrival at the lab. This EDTA blood sample was used for %HbA1c analyses. See section 4.2.3 for the descriptions of the different assays.

4.2.3 Assays

Analyses of blood samples for the different biological markers were as follows:

%HbA1c: The EDTA tube that was frozen immediately upon arrival at the laboratory was transported to the National Health Laboratory Services (NHLS) at the Steve Biko Academic Hospital in Pretoria, South Africa. Here the samples were analysed for HbA1c with a SYNCRON Systems Hemoglobin A1c kit. The kit calculates total haemoglobin concentration by means of a colorimetric method and haemoglobin A1c concentrations by a turbidimetric immunoinhibition method. %HbA1c was calculated by dividing the A1c concentration (g/dL) by the total haemoglobin concentration (g/dL) and multiplying the

answer by 100. This assay, although validated and in use for human diagnostics, has not been validated for lion samples. Only %HbA1c values were obtained from the NHLS.

During the two year period that sampling was conducted, the NHLS made some procedural changes to the HbA1c analysis. This was beyond external user control (in this case, I M Viljoen). This had the unfortunate effect that no results could be obtained from samples analysed after this change. HbA1c results were thus available for only the KNP lions and some of the NZG lions.

Glucose: Frozen serum samples were transported to the Laboratory of Clinical Pathology at the Department of Companion Animal Clinical Studies, Faculty of Veterinary Sciences, Onderstepoort campus, University of Pretoria. Levels of glucose were assayed by means of the Cobas Integra 400 plus analyser (Roche, Randburg, South Africa) using the Cobas Integra Glucose HK reagent (GLUC2) kit. In an effort to do a partial validation of the assay procedure domestic cat samples were also run. These cat samples were obtained from excess samples available from domestic cats that were hospitalised at the Faculty of Veterinary Sciences, Onderstepoort.

Insulin: Frozen Serum samples were transported to the NHLS. Insulin was assayed using a Chemiluminescent immunoassay (Access Immunoassay Systems, Ultrasensitive Insulin, Beckman Coulter, Ref 33410). In addition samples from the same domestic cats used for glucose analyses were analysed for insulin.

Leptin: Frozen Serum samples were used for Leptin analyses. Analyses were done by Prof. N. Bennett at the Department of Zoology end Entomology, University of Pretoria using the Multi-species Leptin RIA kit supplied by Merck-Millipore (Cat. # XL-85K). Validation for lion samples could not be performed, however, a parallelism test and non-linear curve fit analyses were done (Figure 4.1). A parallelism test compares a sample dilution curve against the standard curve of the assay. If the slopes of the sample dilution curve and the standard curve do not differ significantly one can assume that the substance measured in the lion plasma has the same immunological activity as the leptin standard and is therefore likely representative of circulating lion leptin. The parallelism test showed no statistical difference between the standard curve and sample dilution curve (Figure 4.1).This suggests that constituents in lion serum did not interfere with leptin measurements. Therefore, leptin results, while possibly not representing absolute levels, could thus be considered representative of the circulating levels of leptin in the lion blood.
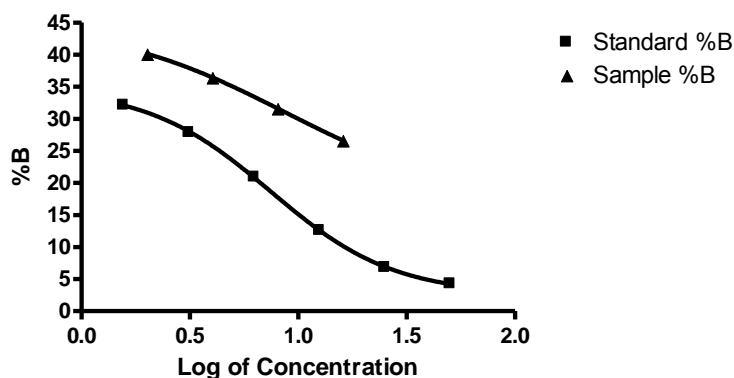
Figure 4.1: Graph of the parallelism test for the sample dilution curve against the Leptin RIA standard curve.

Ghrelin: Frozen serum samples were used for ghrelin analyses. Analyses were done using the Ghrelin (Total) RIA kit supplied by Merck-Millipore (Cat. # GHRT-89HK). Analysis was done according to the supplier guidelines. While validation for lion ghrelin was not done a parallelism test and non-linear regression curve analysis was done (Figure 4.2). This was done with a single sample as well as with a pooled sample (serum pooled from 6 lions). Both the single sample and the pooled sample were double diluted a total of seven times. The resultant dilution curves of the pooled sample and the single sample did not differ significantly. Both the dilution curves did not differ significantly from the standard curve of the assay for standard concentrations ranging from 230 to 3750 pg/ml. It can therefore be assumed that constituents in lion serum did not interfere with ghrelin measurements at concentrations within this range. Therefore, results that fall within this concentration range are most likely representative of ghrelin concentrations in lion serum.
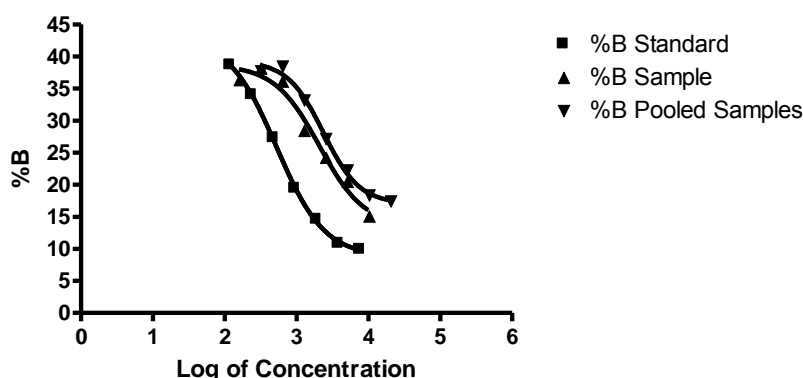
Figure 4.2: Graph of the Ghrelin parallelism test with all values included.

Body Mass Index (BMI): In order to calculate the BMI of lions, certain morphometric measurements were taken - The body length (BL) was measured in meters from the occiput on the back of the head to the base of tail (at point of indentation when tail is lifted vertically);

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Shoulder height (SH) of animal, measured in meters from centre of the metacarpal pad in a straight line to the top of ridge of the scapula (keeping the leg and measuring tape straight and not following the curve of the shoulder); The mass of the animal was measured in kilograms.

The formula to calculate the BMI was BMI = (1/(BL x SH)) x mass[5]

Some lions could not be weighed due to local equipment failure or logistical difficulties and the BMI could, therefore, not be calculated for those individuals.

Note on time since last meal: Many of the energy metabolism markers are sensitive to the time since the last meal. This should preferably be considered when interpreting the relevance of data analyses when comparing different sample cohorts. For the purpose of this study, lions were classified into one of four groups according to the time that passed between the last meal and the time of sampling. The four classifications were: Last meal > 24 hours before sampling; last meal 12 - 24 hours before sampling; last meal < 12 hours before sampling; and time since the last meal unknown. Since the lions sampled in the KNP formed part of a larger demographic study, many of the prides were under surveillance for at most 48 hours prior to the sampling event. It was therefore possible with relative certainty to know if the pride made a kill in the 48 hour period before sampling. Determining when last the captive lions ate was much easier since they were reliant on the keepers for food and where possible feeding of the lions were avoided for the 24 hour period preceding sampling.

4.2.4 Statistical analyses

For statistical analyses and some visualisation of the energy metabolism markers, GraphPad Prism Version 4 for Windows (GraphPad Software, Inc., CA, USA) was used. Either a two-tailed unpaired t-test with Welch's correction (Welch test) was used when variances differed significantly or a two-tailed unpaired Mann Whitney test (MW test) when variances were not significantly different. The Kruskal-Wallis one-way ANOVA test was used for simultaneous comparison of three or more categorical groupings.

Multivariate statistical analyses were done with R using RStudio for Windows (version 0.99.482) (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, http://www.rstudio.com/). Principle component analyses (PCA) were done with the data normalised by means of the "scale" function in R that uses the formula: normalised x = (x – column mean)/column standard deviation). The PCA results were plotted

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[5] The BMI formula and guidelines for measurements were obtained through personal communication with Dr. A. Tordiffe from the National Zoological Gardens in Pretoria who is in the process of developing a BMI for lions.

as score and correlation plots. The different categorical variables were respectively super imposed on the PCA plots. Scatterplot-matrixes were also compiled for the different markers and in some instances distance based cluster analyses were done using all or a selection of the energy metabolism markers.

Packages used in R:

- "base" package – PCA and cluster analyses
- "ggplot" package – Plotting of PCA score and correlation plots
- "car" package – Scatterplot-matrix and spearman correlations

4.3. Results

The concentrations of the different biological markers are summarised in Table 4.1. The mean blood glucose concentration of wild lions in this study was similar to concentrations reported in previous studies. Blood glucose concentrations of captive lions in general were slightly higher than previously reported but similar to that of hospitalised domestic cats (see discussion on glucose assay under section 4.2.3) assayed in the current study. Calculated mean plasma glucose (MPG) and estimated average glucose (EAG) concentrations calculated from %HbA1c were lower than glucose concentrations measured in this study as well as in previous studies. Insulin concentrations in this study were approximately 10 to 100 fold lower than those reported for domestic cats previously (Backus et al. 2007) and in the current study. The insulin concentrations of hospitalised domestic cats in the current study were three to six times lower than previously reported for healthy cats. Leptin concentrations fell within the range reported for domestic cats while ghrelin concentrations fell between the two ranges, but closer to the higher range, previously reported for domestic cats (see Table 4.1).

4.3.1 Time since last meal

The majority of the KNP/wild lions ate more than 24 hours before sampling while the time since the last meal was unknown for the rest. The captive lions were represented in all groups with the majority of lions having eaten more than 12 hours before sampling. Both the probably *M. bovis* infected and uninfected subsets included lions that ate mostly more than 24 hours before sampling or for which the time since the last meal was unknown. Two lions that had a meal between 12 and 24 hours before sampling was also included in the uninfected subset.

4.3.2 Body Mass Index (BMI)

The BMI scores differed significantly (Welch test, p = 0.0028) between wild/*M. bovis* exposed and captive/*M. bovis* unexposed lions (Figure 4.3). Lions of the probably uninfected subset had higher, although not significant (MW test, p = 0.0987), BMI scores than the probably infected subset (Figure 4.3). This may simply reflect an effect owing to captive compared to wild diet, or may also represent a number of other factors.
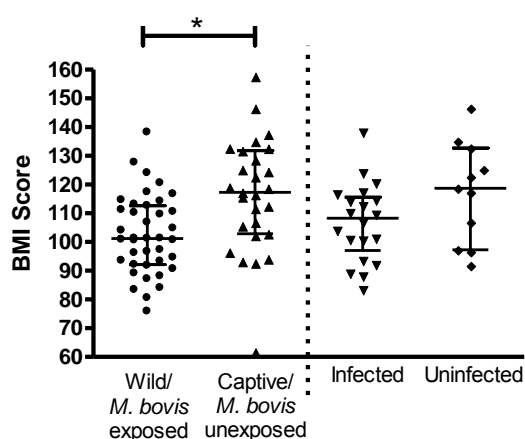
Figure 4.3: Scatter-dot plots of BMI data for the different *M. bovis* exposure groups (left hand side) and the probably infected and uninfected subsets (right hand side). Lines represent median and interquartile ranges. *M. bovis* exposed lions differed significantly (Welch test, *p = 0.0028) from the *M. bovis* unexposed lions

The BMI scores were inspected with relation to the different age classes. In some instances a specific age class was poorly represented in the different lion populations and statistical comparisons could not be done. For both wild and captive lions, juveniles had significantly lower (Wild: MW test, p < 0.0001; captive: MW test, p = 0.0032) BMI scores than adults. Captive sub-adult lions had a significantly (MW test, p = 0.0360) lower BMI score than captive adult lions. The BMI scores of captive juvenile lions, although lower, did not differ significantly (MW test, p = 0.1091) from that of captive sub-adult lions. Due to these age class differences and the small sample size of probably uninfected juveniles and probably infected or uninfected sub-adults, only adult BMI scores of the infected and uninfected subsets were compared. The BMI scores of probably infected adult lions were not significantly lower (MW test, p = 0.0699) than that of their uninfected counterparts. Within the different age classes BMI scores did not differ significantly between genders.

Table 4.1: Summary of biological markers as measured for the different lion populations and for the infected and uninfected subsets. Values given as Mean ± SD, unless stated otherwise.

Biological marker	KNP/Wild/ *M. bovis* exposed	NZG	Ukutula	Captive/ *M. bovis* unexposed	Probably *M. bovis* infected	Probably *M. bovis* uninfected	Reference values*
Glucose mmol/L	5.6 ± 1.9	8.7 ± 1.6	7.1 ± 2.3	7.5 ± 2.3	5.6 ± 1.8	6.2 ± 2.4	5.3 ± 1.6 Asiatic, lion (Jani et al. 2007) 4.2 ± 0.2 to 5.9 ± 0.2 Range, lion (Behera et al. 2013) 3.6 to 14.9 Range, lion (Fahlman et al. 2005) 5.63 Mean, lion (Erasmus, 2008) 3.9 to 4.4 Range of means, cats (Backus et al. 2007) **7.4 ± 1.7 Hospitalised cats, current study**
[a]MPG mmol/L	-	-	-	-	1.6 ± 0.8	1.6 ± 0.7	N/A
[b]EAG mmol/L	-	-	-	-	2.2 ± 0.7	2.2 ± 0.6	N/A
Insulin mIU/L	0.35 ± 0.42	0.05 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.42 ± 0.43	0.08 ± 0.04	3.5 to 6.7 Range of means, cats (Backus et al. 2007) **1.0 ± 0.6 Hospitalised cats, current study**
Leptin ng/ml	2.3 ± 0.9	2.4 ± 1.2	2.1 ± 1.1	2.2 ± 1.1	2.3 ± 0.9	2.4 ± 1.1	2.3 to 2.8 Range of means, cats (Backus et al. 2007)
Ghrelin pg/ml	794.7 ± 208.2	759.1 ± 215	877.9 ± 288.6	808.8 ± 253.1	817.4 ± 246.1	819.8 ± 207.9	220.46 to 287.4 Range of means, cats (Tvarijonaviciute et al. 2012) 1300 to 2000 Range of means, cats (Backus et al. 2007)
%HbA1c	3.04 ± 0.4	2.9 ± 0.3	-	2.9 ± 0.3	2.99 ± 0.4	2.99 ± 0.4	N/A

*Values as reported in the literature. When available, values for lions are given otherwise values reported for domestic cats are given. For studies conducted on different treatment groups the values are given as a "Range of means" showing the lowest and highest mean concentrations reported. N/A -refers to cases where no known values of those biomarkers are available for either lions or domestic cats.

[a]MPG – Mean plasma glucose calculated from %HbA1c as described by (Rohlfing et al. 2002).

[b]EAG – Estimated average glucose calculated from %HbA1c as described by (Nathan et al. 2008).

Since total body fat stores can affect the circulating concentrations of insulin, leptin, and ghrelin (Backus et al. 2007; Meier & Gressner, 2004), these biological markers were inspected in relation to BMI scores. Because of the significant differences between BMI scores of adult and juvenile lions and the small sample sizes of captive, uninfected, and infected juveniles, these analyses were done on data from adult lions only. These results will be presented under the later sections dedicated to each of these markers.

4.3.3 Glucose

Serum glucose concentrations differed significantly (one-way ANOVA, $p < 0.0001$) between lion populations (Figure 4.4, B). Captive lions had significantly higher (MW test, $p = 0.0004$) glucose concentrations than wild lions (Figure 4.4, A). Inspection of the glucose concentrations with regards to the time since the last meal was done. For the wild lions glucose concentrations did not differ between animals that ate more than 24 hours before the time of sampling and animals for which the time since the last meal was unknown. Captive lions that ate more than 24 hours before sampling did not have significantly different glucose concentrations from animals that ate less than 24 or 12 hours before sampling (one-way ANOVA, $p = 0.8418$). However, captive lions for which the time since the last meal was unknown differed significantly from lions that ate more than 24 hours and more than 12 hours before sampling but not from lions that ate less than 12 hours before sampling. Of the lions that ate more than 24 hours before the time of sampling, glucose concentrations in wild lions were significantly lower (MW test, $p = 0.0087$) than in captive lions. This was not the case for lions for which the time since the last meal was unknown. When the glucose concentrations of the probably infected and uninfected subsets were investigated in relation to the time since the last meal, both subsets showed no significant differences between lions that ate more than 24 hours before sampling and lions for which the time since the last meal was unknown. Serum glucose concentrations did not differ (MW tests, $p = 0.5311$) between the subsets of probably *M. bovis* infected or probably *M. bovis* uninfected lions (Figure 4.5). Additionally, glucose concentrations of lions that ate more than 24 hours before sampling did not differ significantly between the infected and uninfected subsets nor did the glucose concentrations differ for lions for which the time since the last meal was unknown.

No associations between glucose concentrations and ITT or GEA results were observed for either wild (*M. bovis* exposed) or captive (*M. bovis* unexposed) lions.
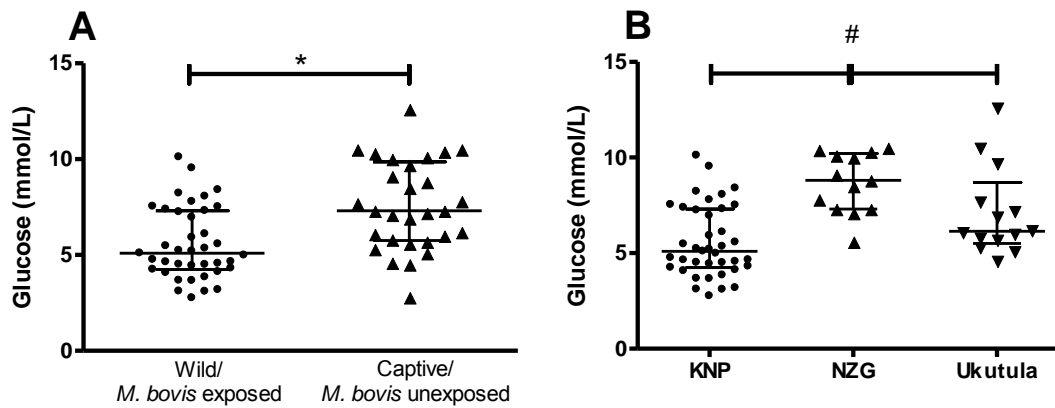
Figure 4.4: Scatter-dot plots of serum glucose concentrations according to the lions' place of origin. Lines represent median and interquartile ranges. A - *M. bovis* exposed lions had significantly (MW test, *p = 0.0004) lower glucose concentrations compared with *M. bovis* unexposed lions. B - The glucose concentrations differed significantly (one-way ANOVA, #p < 0.0001) between the lion populations.
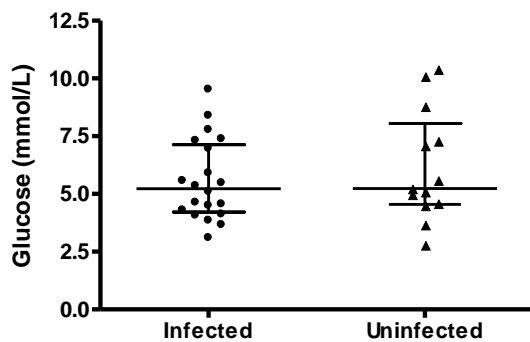
Figure 4.5: Scatter-dot plots of serum glucose concentrations of the probably *M. bovis* infected and uninfected subsets of lions. Lines represent median and interquartile ranges. Glucose concentrations did not differ significantly (MW tests, p = 0.5311) between probably infected and uninfected lions.

The glucose results from the different lion populations and different infection classifications were further divided into specific age classes and/or gender. Many of these subdivisions resulted in small sample sizes for which meaningful statistical analyses could not be done. Glucose concentrations in wild juvenile lions were significantly greater (MW test, p = 0.0294) than in wild adult lions. Glucose concentrations did not differ significantly (one-way ANOVA, p = 0.7900) between the captive age classes. Wild male lions had significantly greater (Welch test, p = 0.0071) glucose concentrations than wild female lions. Further division into age classes showed that the glucose concentrations did not differ significantly between wild adult male and female lions but wild juvenile female lions had

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significantly lower (MW test, p = 0.0097) glucose concentrations than their male counterparts. However, for both wild adult and juvenile lions, small sample sizes of either one or both of the genders brings the meaningfulness of the statistical analyses into question. Glucose concentrations did not differ significantly between genders in the captive lions. Small sample sizes of the probably infected and uninfected subsets prevented any further gender and age based analyses. Only adult female lions could be compared between the infection subsets and their glucose concentrations did not differ significantly.

Nathan et al. (2008) and Rohlfing et al. (2002) proposed linear equations to calculate respectively estimated average glucose (EAG) and mean plasma glucose (MPG) levels from human HbA1c values. Using these equations, both EAG and MPG were calculated from the HbA1c values available for some lions. Neither the calculated EAG nor MPG values could distinguish between lions from the different populations, *M. bovis* exposure groups or suspected *M. bovis* infection groups. However, the MPG was significantly lower (paired t test, p < 0.0001) than the EAG.

4.3.4 Insulin

One KNP lion had an insulin concentration that was more than 10 fold higher than all other lions and was subsequently excluded as an outlier from all analyses. It should be noted that this lion was considered to be probably infected. Note: Unfortunately it was not possible to collect repeat samples for testing to assess whether this was a technical artefact or real result.

Insulin concentrations of captive/*M. bovis* unexposed lions were significantly lower (Welch test, p < 0.0001) than that of wild/*M. bovis* exposed lions (Figure 4.6, A). Because all of the lions grouped in the probably infected subset originate from the wild, comparisons were done with these lions excluded. Even so, insulin concentrations of captive lions were significantly lower (Welch test, p = 0.0160) than that of wild lions (Figure 4.6, A). A comparison of the insulin concentrations of the probably infected wild lions with the rest of the wild lions did not show a significant difference (MW-tests, p = 0.5791). However, two of the wild lions that were not included in the probably infected subset had insulin concentrations that could be considered outliers (encircled points in Figure 4.6, B). Both of these lions were classified as probably infected by means of the GEA while no other diagnostic tests were positive. One of these lions did not have an ITT result. When these two data point were excluded from the analyses the probably infected wild lions had significantly higher (Welch test, p = 0.0263) insulin concentrations than the rest of the wild lions.

Insulin concentrations in the probably infected subset were significantly higher (Welch test, p = 0.0032) than that of the probably uninfected subset. Within these subsets, insulin concentrations were not significantly affected by the time since the last meal. No

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significant differences were observed between insulin concentrations of the different last meal classifications in the wild lions. This was also the case in the captive lions.
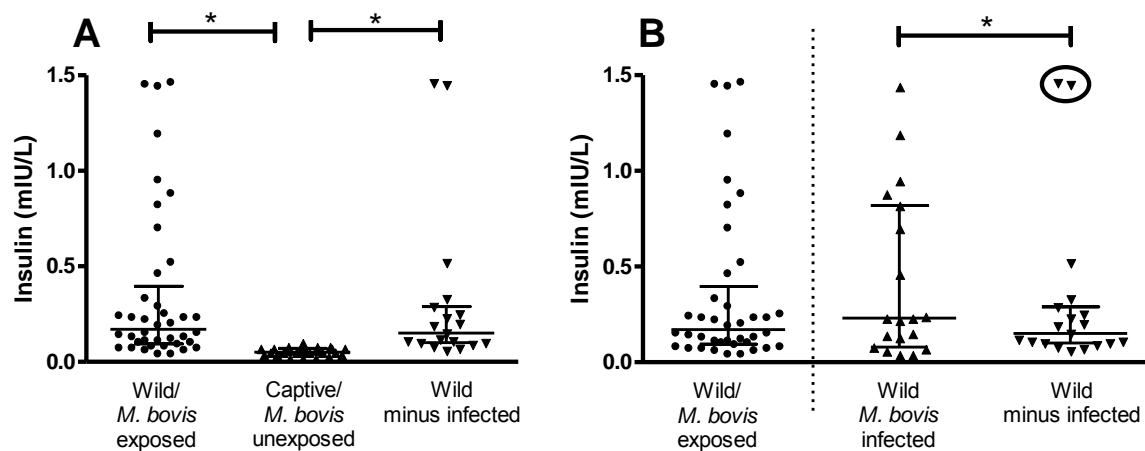
Figure 4.6: (A) Scatter-dot plots of insulin concentrations showing differences between *M. bovis* exposure groups (Welch test, *p < 0.05). (B) Insulin concentrations of the wild lions (Left of the dotted line) were separated into probably infected wild lions and the remainder of the wild lions groups (right of the dotted line). With the encircled outliers removed the insulin concentrations of the wild probably infected lions differed significantly (Welch test, *p < 0.05) from the rest of the wild lions. Solid lines represent median and interquartile ranges.

It was not possible to differentiate between ITT positive and negative lions using the insulin concentrations in wild lions or in captive lions. Inspection of the insulin results obtained for wild lions in light of their GEA diagnostic classification showed that the highest insulin concentrations were present in lions classified as either probably infected or suspect of infection (Figure 4.7).
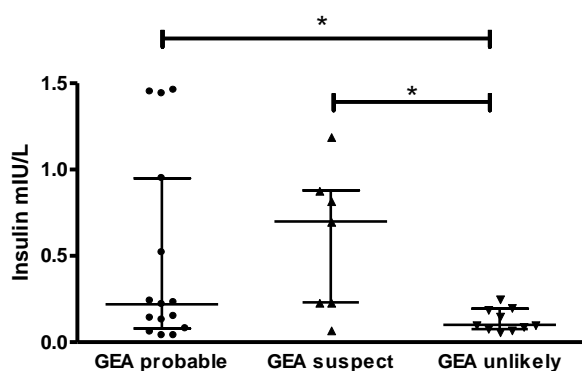
Figure 4.7: Scatter-dot plots of insulin concentrations in wild/*M. bovis* exposed lions grouped according to the GEA diagnostic classification. Solid lines represent median and interquartile ranges. Insulin concentrations of lions classified as unlikely to be infected by means of the GEA differed significantly (Welch test, *p < 0.05) from lions classified with the GEA as probably infected and suspected of infection.

The insulin data were further investigated with relation to age and gender. In some instances this division resulted in small sample numbers and statistical analyses could not be done. The results for the divisions for which the sample size was adequate are as follows:

Wild juvenile lions had significantly higher (MW test, p = 0.0044) insulin concentrations than wild adult lions. Captive juvenile lions had significantly lower (MW test, p = 0.0482) insulin concentrations than captive adults. However, the small sample size of captive juveniles (n = 4) together with the low power brings the true significance of this into question. Insulin concentrations of captive sub-adult lions did not differ significantly from either adult or juvenile captive lions. Wild female lions had significantly lower (MW test, p = 0.0126) insulin concentrations than wild males, however, insulin concentrations did not differ between the genders in captive lions. Insulin concentrations did not differ significantly between adults of the probably infected and uninfected subsets.

Linear regressions and correlations were done with the BMI scores and insulin concentrations for adult lions from the different exposure groups and the different infection classifications. In general, insulin concentrations increased with increased BMI. The strongest correlation was observed in the uninfected subset (Spearman's, r = 0.7488), with a weaker correlation (Spearman's, r = 0.6530) in the infected subset (Figure 4.8). Correlation coefficients (Spearman's r) for *M. bovis* exposed and unexposed lions were respectively 0.5667 and 0.5509.
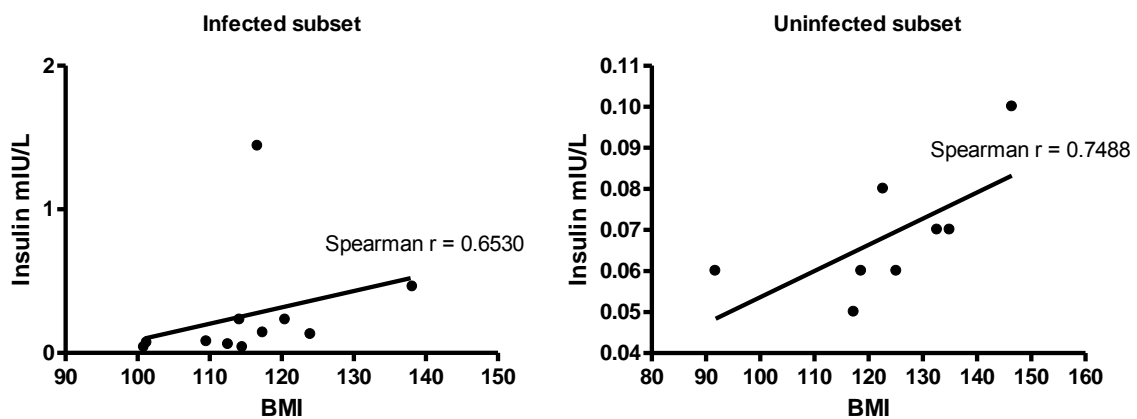
Figure 4.8: Linear regressions of BMI scores and insulin concentrations for the infected (left) and uninfected (right) subsets. Spearman's correlation coefficient indicated.

4.3.5 HbA1c

As mentioned in section 4.2.3, %HbA1c was measured in the KNP lions and some NZG lions only. The %HbA1c values were not able to distinguish between KNP and NZG lions, the probably infected and uninfected subsets (Figure 4.9, A), ITT positive and negative KNP lions, or between the GEA classes of the KNP lions. No differences were observed

between KNP lions that ate more than 24 hours before sampling or lions for which the time since the last meal was unknown (Figure 4.9, B).
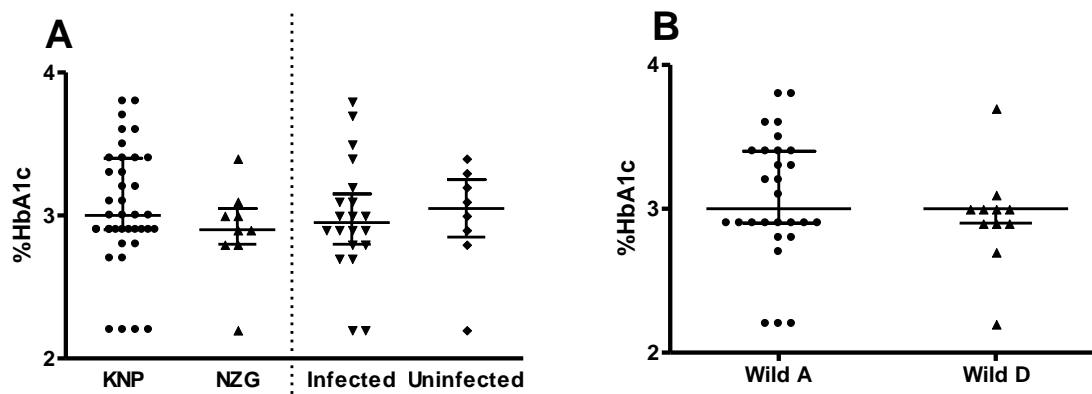
Figure 4.9: (A) Scatter-dot plots of the %HbA1c values with regards to the sampled lion population (Left of dotted line) and with regards to the probability of *M. bovis* infection status (right of dotted line). (B) Comparison between the last meal groupings of wild lions (Wild A = last meal > 24 hours before sampling, Wild D = time of last meal unknown). Solid lines represent median and interquartile ranges. None of the %HbA1c values differed significantly within any of the population, probability of infection, or last meal groupings.

4.3.6 Leptin

Leptin concentrations were not affected by the time since the last meal. Leptin concentrations were not distinguishable between different lion populations, *M. bovis* exposure groupings, or *M. bovis* infection classifications. The only significant difference (MW test, p = 0.0348) was observed between leptin concentrations of wild ITT positive lions and wild ITT negative lions (Figure 4.10).
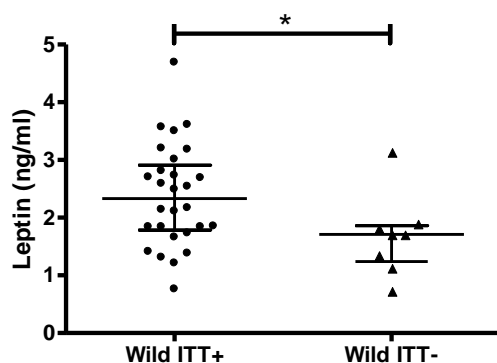
Figure 4.10: Scatter-dot plots of leptin concentrations in wild lions grouped according to ITT result. Solid lines represent median and interquartile ranges. Leptin concentrations in wild ITT positive lions were significantly greater (MW test, *p < 0.05) than in wild ITT negative lions.

Leptin concentrations in juvenile lions were significantly lower than adult lions for both wild (MW test, p = 0.0130) and captive (MW test, p = 0.0177) populations. The leptin concentrations of captive sub-adult lions were similar to that of captive adult lions. Probably infected juvenile lions had significantly lower (MW test, p = 0.0276) leptin concentrations than probably infected adult lions. Comparisons between captive and wild lions did not show differences in leptin concentrations for either adult or juvenile lions. The leptin concentrations did not differ significantly between adult lions of the infected and uninfected subsets.

An attempt was made to look at possible gender based differences in leptin concentrations. However, due to the age related differences any further subdivisions resulted in small sample sizes not suitable for meaningful statistical analyses.

Linear regressions of leptin concentrations with BMI scores of adult lions showed a general increase in leptin with an increase in BMI. Only for the lions from the infected subset did leptin and BMI correlate (Spearman r = 0.6503). The ratio of leptin/BMI was calculated and compared between the *M. bovis* exposure groups and between infection groups. No significant differences were observed.

4.3.7 Ghrelin

As mentioned in section 4.2.3, blood ghrelin concentrations that fall within the range of 230 to 3750 pg/ml can be considered representative of the circulating ghrelin concentrations in lions. None of the ghrelin concentrations in this study fell outside this range. The time since the last meal did not affect ghrelin concentrations in lion blood. Ghrelin concentrations did not differ between the different lion populations, *M. bovis* exposure groups, or probably infected and uninfected subsets, nor were any age related differences observed between or within the exposure or infection groupings. Additionally, ghrelin concentrations were not able to distinguish between ITT positive and ITT negative lions or between lions in the different GEA classification groups.

Ghrelin concentrations did not differ significantly between male and female lions within the different *M. bovis* exposure groups or infection classifications. Comparisons of ghrelin concentrations between the same genders of the different exposure groups and infection classifications did not yield any significant differences.

Linear regression analyses showed a general decrease in ghrelin concentrations with an increase in BMI for the adult lions of the *M. bovis* exposed group and both the infected and uninfected subsets (Figures 4.11 and 4.12) while a general increase in ghrelin was observed with an increase in BMI in the captive/*M. bovis* unexposed lions (Figure 4.11). None of the ghrelin concentrations and BMI scores correlated for any of the *M. bovis* exposure groups or infection classifications.

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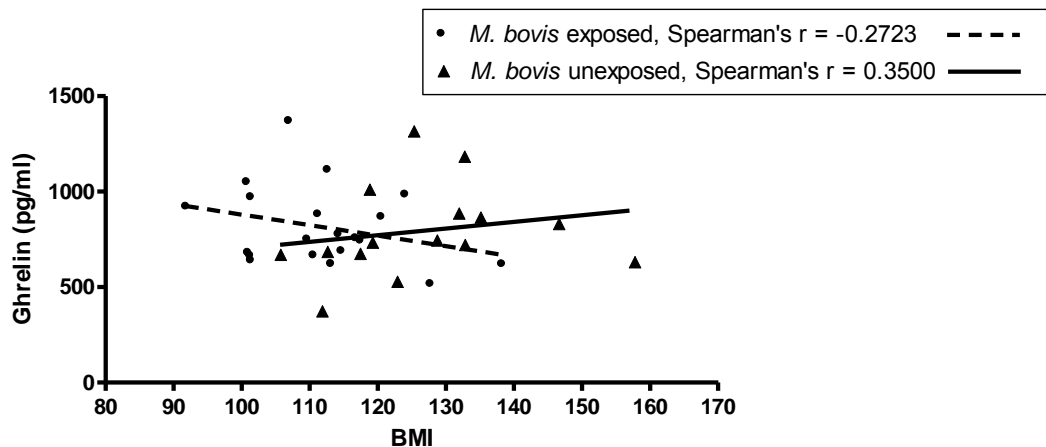
Figure 4.11: Linear regressions of BMI scores and ghrelin concentrations for the adult lions of the two *M. bovis* exposure groupings. Spearman's correlation coefficient indicated in the key.
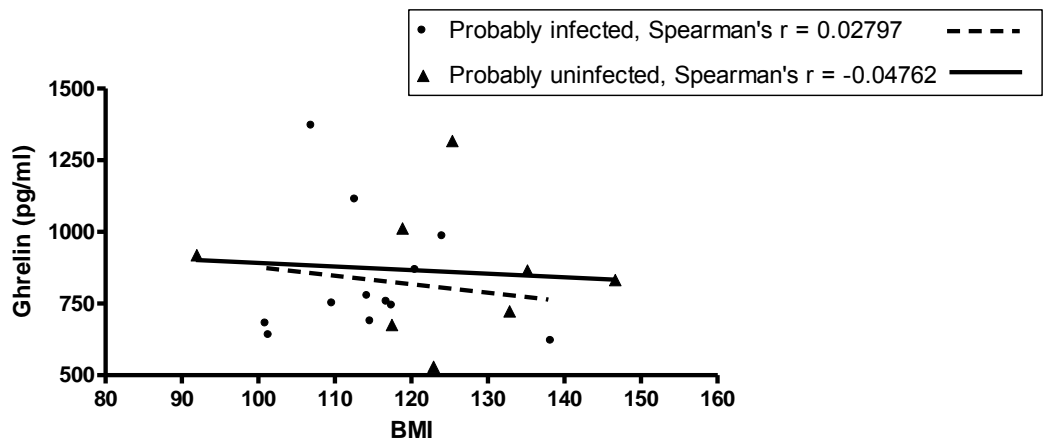
Figure 4.12: Linear regressions of BMI scores and ghrelin concentrations for the adult lions according to probability of *M. bovis* infection. Spearman's correlation coefficient indicated in the key.

4.3.8 Multivariate analyses of metabolic markers

From the above results, age related differences were observed for BMI scores and for insulin, glucose, and leptin concentrations. The majority of age related differences were between adult and juvenile lions with the exception of the BMI scores where captive sub-adult scores also differed significantly from adult scores. Additionally, within the probably infected and uninfected subsets, juvenile and sub-adult lions were poorly represented. Therefore, in addition to multivariate analyses with the complete data sets of the exposure and infection groupings, analyses were also done with data from only the adult lions. Biological markers included in the multivariate analyses were: glucose, insulin, leptin, ghrelin, and BMI. Due to poor representation of captive lions and therefore uninfected lions,

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%HbA1c was excluded from the analyses. The lion with the insulin concentration that was considered an outlier was also excluded from the analyses.

Scatterplot matrix diagrams were compiled with the metabolic marker data of all the lions, all the adult lions (Figure 4.13), the probably infected and uninfected lions, and the probably infected and uninfected adult lions (Figure 4.14). In all cases the general trends were: decreased glucose concentrations with increased insulin concentrations; increased glucose concentrations with an increase in leptin concentrations as well as with an increase in BMI score; and increased leptin concentrations with an increase in BMI score. None of the Spearman's r scores for any of the data sets exceeded 0.5. In the data set comprised of all the adult lions, the strongest correlations were observed between glucose and insulin (Spearman's r = 0.43) and glucose and BMI (Spearman's r = 0.42), and was higher than when juvenile and sub-adult data were included. For the probably infected and uninfected adult lions the strongest correlation was observed between glucose and leptin (Spearman's r = 0.42). This was also higher than observed when juvenile and sub-adult lions were included in the data set.
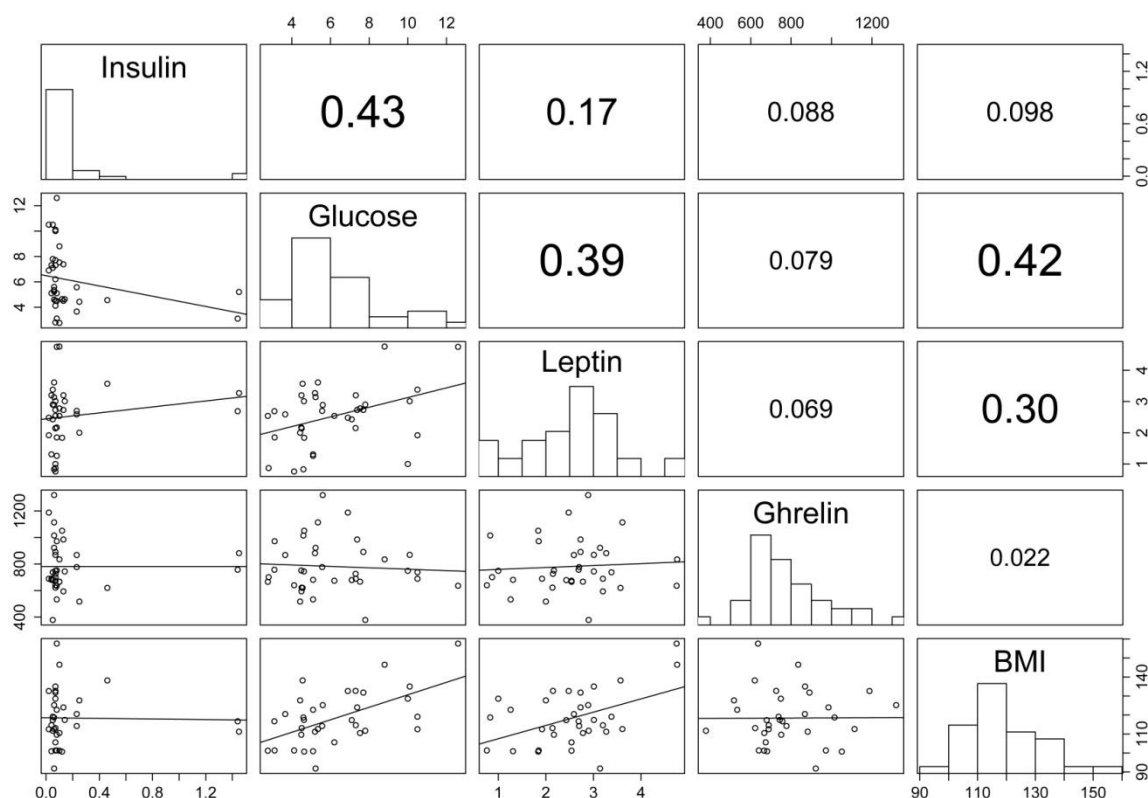
Figure 4.13: Scatterplot matrix of the energy metabolism markers of all the adult lions. Linear regression and Spearman's correlations are between the different markers also indicated.
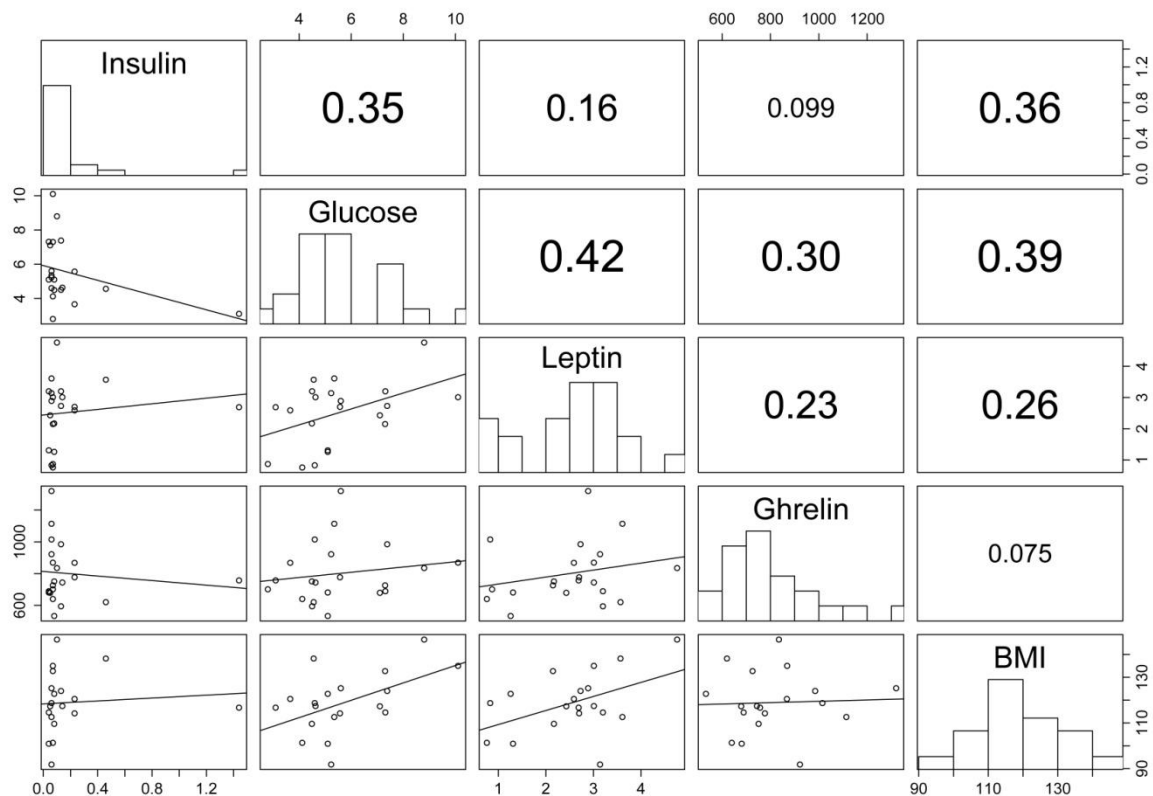
Figure 4.14: Scatterplot matrix of the energy metabolism markers of the probably infected and uninfected adult lions. Linear regression and Spearman's correlations are between the different markers also indicated.

Because of the known associations between leptin and insulin (Woods et al. 1998), and insulin and glucose (Garrett & Grisham, 2005), these markers were inspected at a finer scale.

No correlation was observed between leptin and insulin concentrations in any of the *M. bovis* exposure groups or the infection classifications. This was also the case when only adult data were considered.

Linear regression analyses and correlations between insulin and glucose were investigated. Glucose concentrations were plotted against log Insulin concentrations (Figure 4.15). Insulin and glucose concentrations did not correlate in any of the *M. bovis* exposure groups or the infection classifications. For the captive/*M. bovis* unexposed and the uninfected subset lions, glucose concentrations in general were lower at higher insulin concentrations. Neither this, nor the reverse was seen in wild/*M. bovis* exposed or the infected subset of lions.

Glucose concentrations plotted against log insulin concentrations in adult lions also did not correlate in the different exposure groups or infection classifications. Furthermore, the general trends of decreased glucose with increased insulin in the unexposed and

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uninfected groups were reduced for adult lions (Unexposed adult, Spearman's r = -0.1668; uninfected adult, Spearman's, r = -0.08077). However, this trend became more pronounced in the exposed and infected adult lions (Exposed adult, Spearman's, r = -0.2278; Infected adult, Spearman's, r = -0.4749).
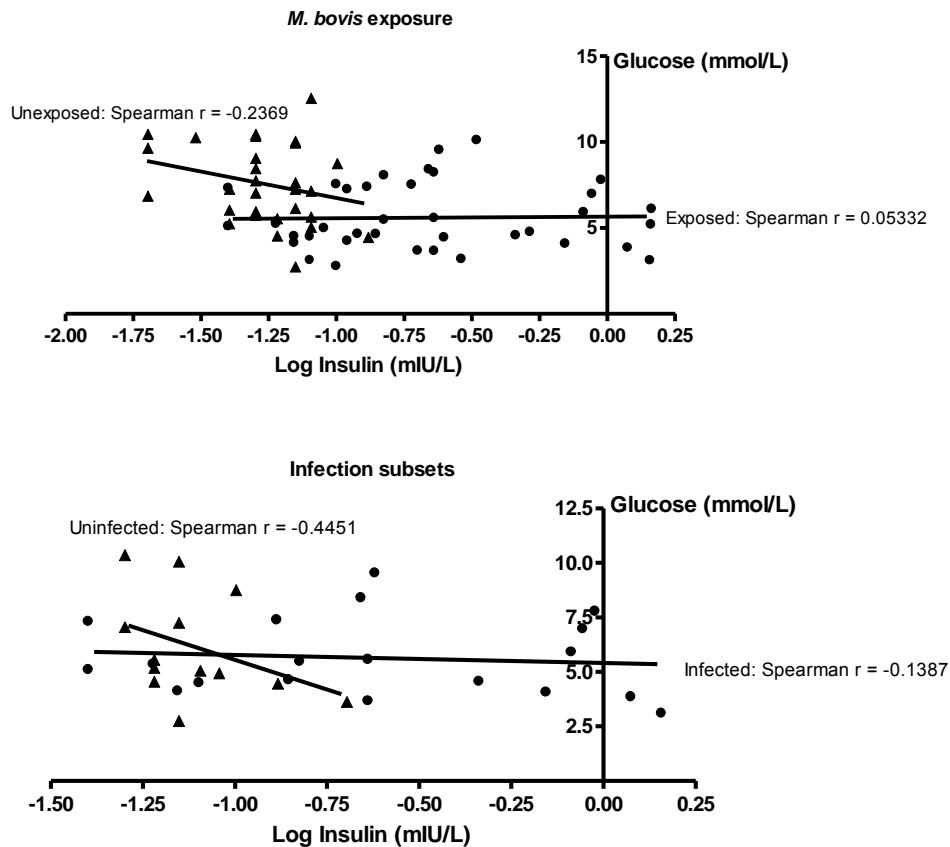
Figure 4.15: Linear regression plots of glucose and log insulin concentrations for the *M. bovis* exposure groups (top) and the probably infected and uninfected lions (bottom). Spearman's correlation coefficients are indicated.

A summary of the PCA's for the different data sets can be seen in Table 4.2. The first two principle components of the data sets comprising of all the adult lions ("All adult"), the probably infected and uninfected lions ("Subset"), or the probably infected and uninfected adult lions ("Subset adult") were able to explain more than 60% of the variance in the data sets. BMI was a major contributor to the first principle component in all the data sets with insulin being the main contributor to the second principle component in all data sets. Both leptin and glucose in many instances contributed a large part to the first component while ghrelin featured twice as a contributor to the second component.

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Score and correlation plots of the PCA results for the different data sets did not show clear differentiation between lions from the various exposure groups or the different infection groups (Figures 4.16 and 4.17). Consideration of only the adult lions did not facilitate a clearer picture. In general, lions from the *M. bovis* exposed area and the probably infected subset were associated more with increased insulin concentrations while unexposed lions and probably uninfected lions were associated more with increased BMI scores and increased leptin and glucose concentrations

Table 4.2: Summary of the PCA results for the first two principle components (PC1 and PC2) of the energy metabolism markers. Data sets used were all the lions ("All"), adult lions ("All adult"), probably infected and uninfected lions ("Subset"), and the adult lions of the probably infected and uninfected subsets ("Subset adult"). Values in bold indicate the biological markers that contribute the most to the relevant principle component.

	All		All adult		Subset		Subset adult	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Proportion of variance	0.33	0.22	0.39	0.24	0.38	0.24	0.40	0.25
Insulin	0.38	**-0.58**	0.11	**-0.79**	0.40	**-0.53**	0.18	**-0.83**
Glucose	-0.51	-0.09	**-0.60**	0.30	-0.47	-0.03	**-0.59**	0.27
Leptin	**-0.54**	-0.26	-0.51	-0.43	**-0.54**	-0.29	**-0.53**	-0.34
Ghrelin	0.05	**0.77**	0.02	-0.30	-0.13	**0.76**	-0.24	0.16
BMI	**-0.54**	0.01	**-0.61**	-0.10	**-0.55**	-0.25	**-0.54**	-0.30
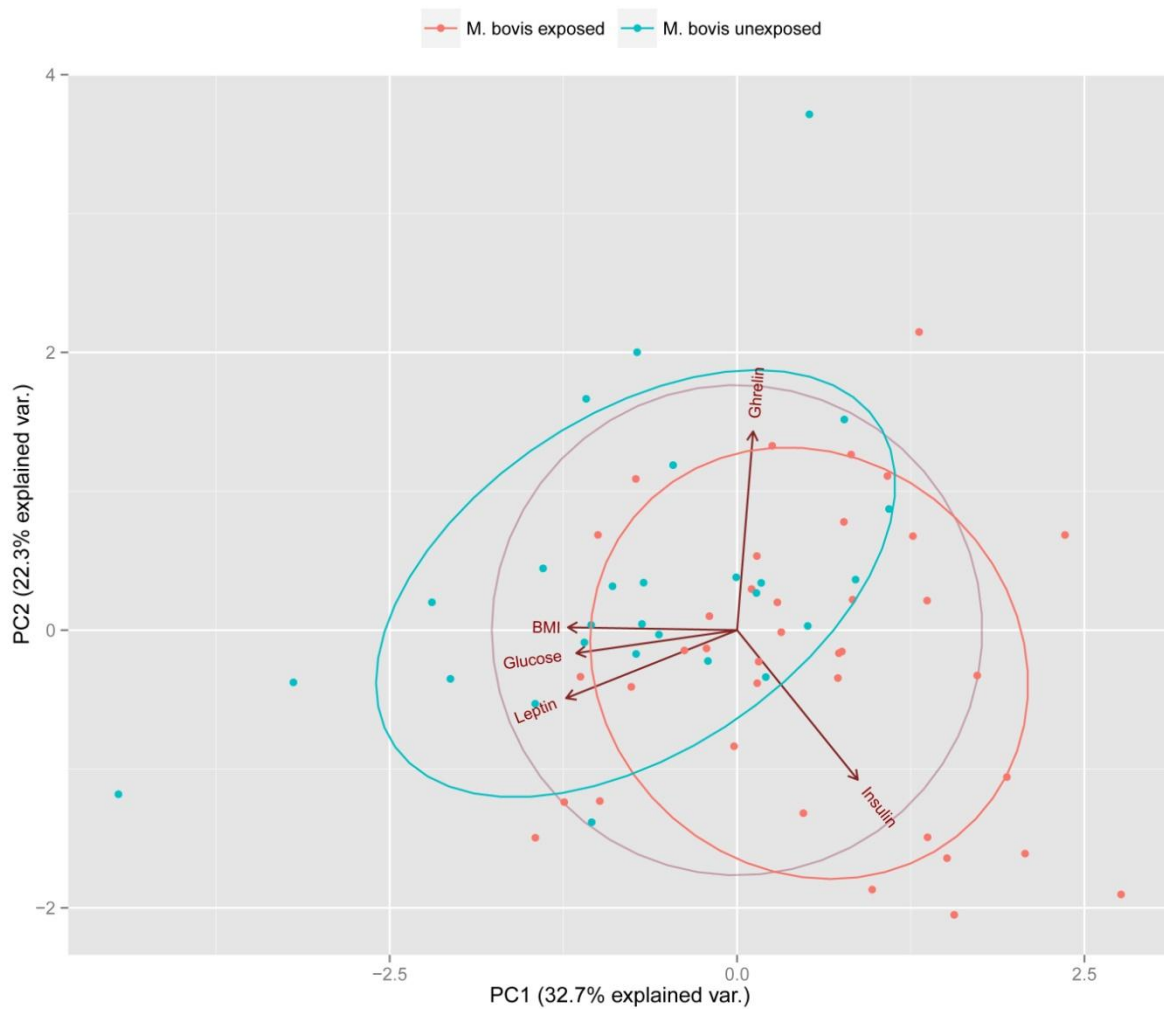
Figure 4.16: Score and correlation plot for the energy metabolism markers of all the lions. Different colours indicate different *M. bovis* exposure groups.
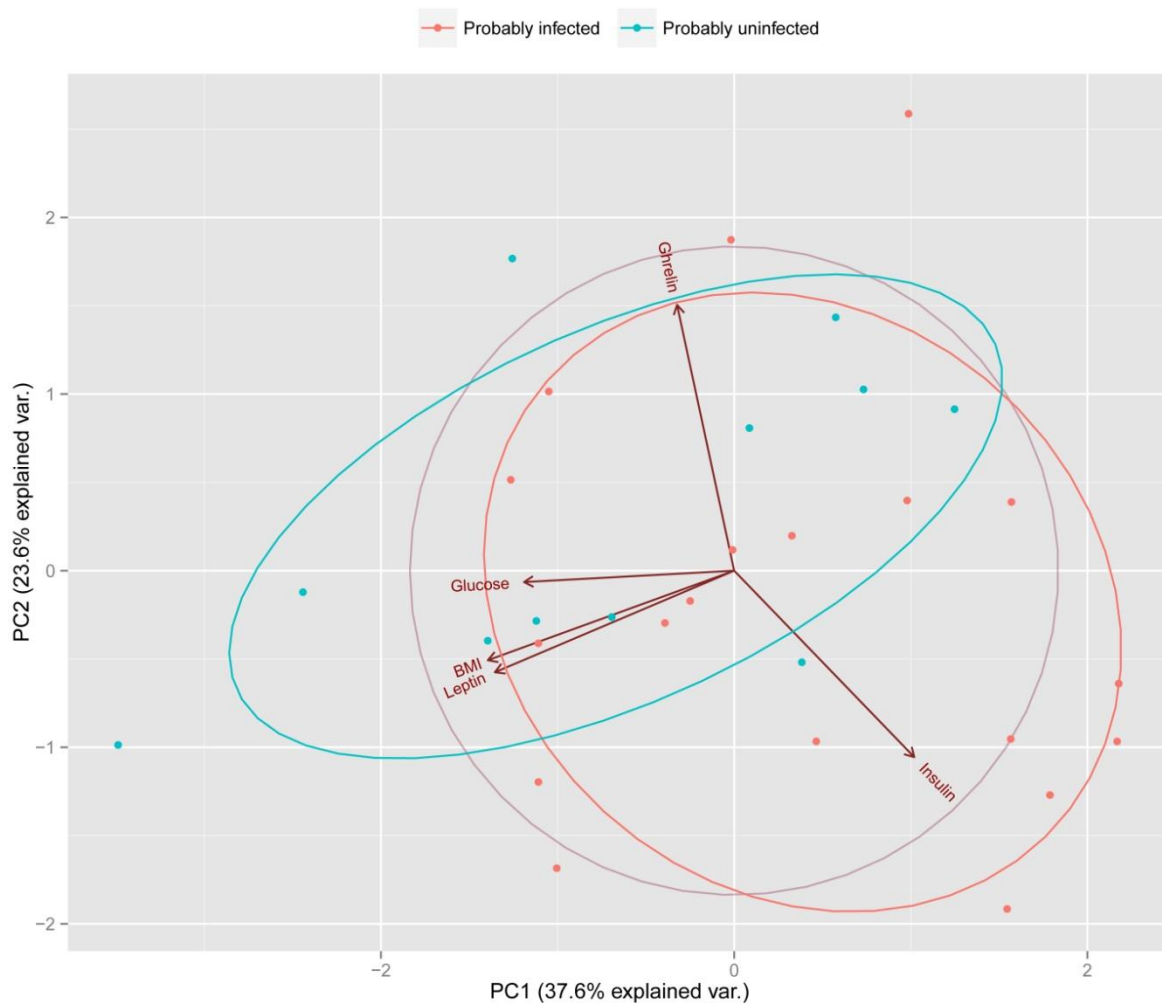
Figure 4.17: Score and correlation plot of the energy metabolism markers for the probably infected and uninfected lions. Different colours indicate different *M. bovis* exposure groups.

Distance based cluster analyses were done with the energy metabolism markers of the different data sets and cluster dendrograms drawn. Analyses with all the markers were not able to successfully group the lions according to their probable infection or *M. bovis exposure* status. Similar results were obtained using only data from adult lions.

From the results described earlier in this chapter the markers leptin, insulin and BMI were selected and distance based cluster analyses were done. Again the different exposure groups could not be separated and the same applied to the different infection groups.

The markers that featured prominently in the first principle components of the PCA results for the different data sets were selected and subjected to distance based cluster analyses. This could also not separate out the different exposure or infection groups.

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4.4. Discussion

Various biological markers may be investigated to gain insights into the state of energy metabolism or homeostasis of an animal. Comparisons of these markers between healthy and *M. bovis* infected or diseased lions might shed some light on the possible metabolic burden experienced by diseased/infected lions. The current study investigated %HbA1c, BMI, and concentrations of glucose, insulin, leptin, and ghrelin in lions that are exposed to *M. bovis* and "presumably unexposed" lions. Furthermore, subsets of probably infected and probably uninfected lions were selected (using criteria described in Chapter 2, section 2.5) for a more detailed comparison between healthy and diseased/infected lions. It should, however, be mentioned that the infection/disease state of these subsets was not confirmed through necropsy

From the literature it is clear that many, if not all, of the markers investigated in this study are to some degree interacting with and affecting one another. I will therefore firstly discuss the results obtained for each marker independently after which some of the observed interactions or lack thereof will be discussed.

In addition to possible effects of disease on the markers, some of them such as insulin, glucose, and ghrelin may be affected by the time since the last meal. All these markers, including leptin were inspected for possible differences between animals for which the time since the last meal differed.

4.4.1. Blood Glucose

As for all mammals, the main source of metabolic energy in lions is glucose (Behera et al. 2013). In addition to the single point measurement of blood glucose concentrations, %HbA1c was also investigated to gain insights into blood glucose concentrations over an extended time period.

4.4.1.1 Glucose

Mean glucose concentrations of wild lions in the current study were in many cases similar to concentrations previously reported for lions while the mean concentration in captive lions was slightly higher. However, the mean glucose concentration of captive lions was similar to that of hospitalised domestic cats, additionally assayed in the current study

Initial inspection of glucose concentrations showed that captive lions had significantly higher glucose concentrations than wild lions. Because the time since the last meal could possibly affect circulating glucose concentrations, the glucose concentrations were inspected while accounting for this. Unexpectedly, the time since the last meal, as classified in this study (section 4.2.3) did not seem to affect circulating glucose concentrations. We may hypothesize that the lower concentrations in the wild/*M. bovis* exposed lions compared to the captive/unexposed lions could possibly be an indication that *M. bovis* is affecting

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glucose concentrations. However, the fact that glucose concentrations did not differ between the probably infected and uninfected subsets does not support this hypothesis. One could speculate that because all of the wild lions were darted while on a baited carcass, that they have technically eaten between half an hour and an hour before sampling while the majority of captive lions had been fasting for at least 12 hours. To my knowledge no data is available with regards to glucose fluctuations in lions in response to a meal, complicating the ability to properly interpret the results. It could be speculated that if wild lions had eaten recently, that their glucose concentrations should be higher than that of captive lions. Alternatively, that a higher insulin concentration should accompany the lower glucose concentrations in response to the preceding increased glucose concentrations.

Studies on domestic cats showed post-prandial increases in blood glucose concentrations within two hours after ingesting a meal, but this was reliant on the proportion of carbohydrates present in the diet with high carbohydrate diets resulting in greater increases in glucose than diets low in carbohydrates or high in starch or protein (Appleton et al. 2004; de-Oliveira et al. 2008). Another study by Martin & Rand (1999) reported no post-prandial hyperglycaemia within and after two hours after a meal. They mentioned that carbohydrate digestibility of cat foods decrease with increased protein content in the food, and ascribed this as a possible reason for the lack of post-prandial hyperglycaemia in their study (Martin & Rand, 1999). Therefore, a possible explanation for the higher glucose concentrations in the captive lions might be due to differences in diet and feeding frequency between captive and wild lions. While speculative, the fact that captive lions receive whole chickens on a frequent basis might be a contributing factor to the higher glucose concentrations in captive lions since the gut contents of whole chickens most likely contain a higher proportion of refined carbohydrates than would be present in wild prey.

Varying results have been reported in previous studies with regards to glucose concentrations in lions. Some studies reported seasonal, gender, and age related differences while other studies did not. The study by Behera et al. (2013) reported higher mean glucose concentrations in the winter for females compared to males. Additionally, they reported a general decrease in glucose concentrations in males from summer to winter with the reverse in females (Behera et al. 2013). In the current study the majority of captive lions were sampled in early to late summer (October to April) while all the wild lions were sampled in mid to late winter (June to August). With the majority of wild lions being females and a greater percentage of captive lions being males, higher glucose concentrations, according to results from Behera et al. (2013), should be expected in the wild population compared to the captive population, which was not the case. Additionally, the current study showed that in the winter months, wild male lions had higher glucose concentrations than females. Age related differences were observed in the wild lions, with higher glucose concentrations in juveniles

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than in adults. Comparisons between genders of the wild lions within these age classes showed no significant differences between adult males and females while juvenile males had significantly higher concentrations than juvenile females. Caution should be aired in interpretation of these results since the sample sets with age and gender accounted for consisted of relative small sample sizes. In the captive population no significant differences were observed between age classes, genders, or genders of the same age class. Furthermore, this data does not address the point of whether such differences are clinically important or not.

Despite the variation of results obtained for glucose concentrations within and between wild and captive lions with regards to age, gender, and season, comparison of the probably infected with the uninfected subset were done and showed no significant differences in glucose concentrations when age and gender where not accounted for. Unfortunately, when age and gender were accounted for comparisons could only be done between adult females of the probably infected and uninfected subsets and their glucose concentrations did not differ significantly.

Taking all of the above into consideration it would seem that the lower glucose concentrations in the wild lions are not due to *M. bovis* infection/disease. This appears to be in agreement with Yurt et al. (2012) who did not find significant differences in glucose concentrations between healthy humans and TB patients.

4.4.1.2 %HbA1c

For unknown reasons, the HbA1c assay (developed for human samples) failed after changes were made to the protocol at the NHLS. Since NHLS is not set up for animal work and our access was limited, we could not repeat or extend this part of the work. Therefore the sample size of lions for which %HbA1c values are available is limited.

One aspect that needs to be kept in mind when inspecting the %HbA1c values is the fact that in humans, factors such as ethnicity can influence %HbA1c (Lyons & Basu, 2012) suggesting that interspecies differences likely exist and it may therefore not be advisable to use human Hba1c guidelines to quantitatively interpret results from lions.

In the current study %HbA1C did not differ between wild and captive lions nor between the probably infected and uninfected lions. Assuming that the %HbA1c values are indeed representative of the situation in lions, this would suggest that the mean blood glucose concentrations, over an extended time period, did not differ between the captive and wild lions or the infected and uninfected lions.

Since no data is available on %HbA1c in lions, I attempted to calculate the estimated average glucose (EAG) concentrations and the mean plasma glucose (MPG) concentrations using formulas presented in human studies. Neither the EAG nor the MPG was able to

distinguish between the different lion populations, exposure, or infection groups. However, the MPG was significantly lower than the EAG. Both the calculated MPG and EAG concentrations were lower than the mean serum glucose concentrations measured in a single point blood sample. This may show that the higher glucose concentrations in the blood samples are due to processes associated with the sedation of the lions or that the analysis of %HbA1c in lion blood using a human test was not reliable.

4.4.2 Insulin

The drug Medetomidine, used in the drug cocktail approved to sedate lions has been shown to decrease insulin concentrations in canine serum samples as early as 20 minutes after administration (Burton et al. 1997). In domestic cats Medetomidine also resulted in a decrease of insulin concentrations with the lowest concentrations measured at one hour after administration (Kanda & Hikasa, 2008). In the current study the majority of blood samples were taken within one hour after administration of the drugs and insulin production was probably still under the inhibitory effect of Medetomidine. The insulin concentrations measured, although not absolute, might still be representative of circulating insulin since higher initial insulin would result in higher insulin concentrations at the time of measurement. Not all lions received the same dose of Medetomidine nor were they sampled at exactly the same time after administration of Medetomidine, thereby lessening the usability of the measured insulin concentrations. However, since the majority of lions were sampled in the same approximate time window after sedation it might still be worthwhile to do further investigation into the insulin concentrations measured in the current study. (Note: knowledge of the possible complications in insulin measurements caused by Medetomidine was obtained after sampling was initiated and while researching publications in order to better interpret the results obtained. Furthermore, the current study connected in with a larger study that was already underway in the KNP in which the use of Medetomidine to sedate the lions was part of the approved protocol and it would not have been possible to change the standard operating procedure (SOP) at the time we joined this study, even if we had knowledge of the possible complications beforehand).

Lion insulin concentrations in the current study were between 10 and 100 times lower than previously reported for domestic cats and approximately 14 times lower than for hospitalised domestic cats in the current study. This might be due to the inhibitory effect of Medetomidine on lion insulin production. However, no data are available on the drugs used to anaesthetise the domestic cats incorporated in the current study that also had slightly lower insulin concentrations than previously reported for healthy domestic cats (See Table 4.1). The lower insulin concentrations could also be due to the use of a human insulin assay and not a lion or feline specific assay.

There is some speculation that insulin resistance can serve as a marker and potential risk factor for active *M. tuberculosis* infection in humans (Mao et al. 2011). Although this data provides no strong evidence, it allows for speculation that in lions one may consider the following: 1) Assume that the insulin concentrations measured in the current study are at least representative of circulating insulin; 2) The highest insulin concentrations were measured in the *M. bovis* exposed lion population (Figure 4.6); 3) Except for two lions, the majority of high insulin concentrations were included in the probably infected subset (Figure 4.6); 4) Captive/unexposed lions as well as wild probably uninfected lions had significantly lower insulin concentrations than probably infected lions; 5) The wild lions with high insulin concentrations were classified as suspect or probably infected by means of the GEA. However, the higher insulin concentrations in the wild lions might also be an artefact of the effect of Medetomidine inhibition starting to wear off since the time from darting to sampling in general was longer for wild lions compared to captive lions.

Similar to what was seen in the glucose results, gender and age related differences in insulin concentrations were predominantly observed in wild lions. Except for lower insulin concentrations observed in captive juvenile lions compared to captive adults, no other age or gender based differences were observed in captive lions. However, the small sample size (n = 4) of captive juvenile lions brings this observed difference into question.

4.4.3 Leptin

The role of leptin in human TB is still debated with some studies reporting lower leptin concentrations in TB patients (Santucci et al. 2011; van Crevel et al. 2002) and others reporting higher concentrations (Çakir et al. 1999). Additionally, some studies report decreased leptin concentrations in association with chronic stimulation of pro-inflammatory cytokines (Santucci et al. 2011; van Crevel et al. 2002).

Leptin concentrations in the lions in the current study compare well to those of domestic cats. The fact that ITT positive lions had lower leptin concentrations than ITT negative lions might be an indication that lions possibly infected with *M. bovis* are experiencing reduced leptin production. This was not substantiated when leptin concentrations were compared between probably infected and uninfected lions. Age related differences were observed for leptin concentrations with juvenile lions having lower leptin concentrations than adult lions. This was the case for captive, wild, and infected lions (sample size of uninfected lions were too small) and is possibly in line with findings described in Meier & Gressner (2004) where leptin concentrations in children and adolescents were age dependent. Accounting for the age related differences, leptin concentrations still did not differ between the wild/exposed and captive unexposed lions.

Looking at leptin alone it does not seem as though *M. bovis* is causing substantial changes to the ability of lions to store energy. However, leptin concentrations can be influenced by factors such as insulin, pro-inflammatory cytokines, and body fat mass and these associations will be discussed in section 4.4.5. Additionally, since the assay used for leptin analyses has not been specifically validated for lions, the possibility still exist that the results obtained are not quantitatively representative, despite parallelism tests indicating that it might be representative of circulating leptin concentrations.

4.4.4 Ghrelin

The role of ghrelin is to a large part the opposite of leptin. In humans it has been reported to increase up to two fold just before a meal and decrease rapidly to its lowest point within an hour after eating. No differences in ghrelin concentrations were observed between lions that ate less than 12 hours or immediately before the time of sampling and lions that ate more than 12 hours before sampling. Additionally, ghrelin levels have been reported to increase in individuals with a negative energy balance and decrease in individuals with excess fat stores. One could therefore anticipate that diseased or energy deprived lions should express higher levels of ghrelin than healthy lions, similar to what is seen in human TB patients. This was not the case in the current study with non-significant differences observed in ghrelin concentrations between infected or uninfected lions.

It might therefore be speculated that *M. bovis* infection in lions is not causing a negative energy balance. However, the ghrelin RIA has not been validated for lions and the above speculation remains just that. Despite this, the ghrelin concentrations of the current study fell within the ranges of ghrelin previously reported for cats, suggesting some relevance to the assumption made.

4.4.5 Multivariate analyses

Age related differences were observed in glucose, leptin, and insulin concentrations. Therefore, in addition to multivariate analyses with all the age classes grouped together analyses were also done with data from adult lions only. Due to a poor representation of the younger age classes in the infected and uninfected subsets analyses with data from juvenile or sub-adult lions were not done.

It has been well documented that strong associations occur between glucose and insulin. In the current study a weak negative correlation was observed between insulin and glucose concentrations when looking at the data from all the lions together. Separating the samples into *M. bovis* exposure groups and infection groups showed no correlation of insulin and glucose in the exposed lions and the infected lions with very weak negative correlations in the unexposed and uninfected lions. This is not in line with what is expected under normal circumstances where increased glucose should result in increased insulin production.

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However, the general trends observed when captive and wild lions were compared (i.e. Higher glucose and lower insulin in captive lions than in wild lions) might be a result of the effects of the drug Medetomidine. Kanda & Hikasa (2008) reported decreased insulin and increased glucose concentrations as a result of Medetomidine administration. In general the time delay between administering Medetomidine and sampling the lions was greater for wild lions than for captive lions, therefore opening the possibility that the inhibitory effects of Medetomidine on wild lion insulin production might have already been wearing off. The differences observed between wild and captive lions, while speculative, could therefore possibly be an artefact of the Medetomidine used in the anaesthesia of the lions.

An alternative explanation for the abnormal findings for glucose in relation to insulin might be found in stress induced hyperglycaemia due to restraint (Rand et al. 2002). The study by Rand et al. (2002) found that increased glucose concentrations in domestic cats were associated with lactate produced in response to the cats' efforts to escape when handled. In their study glucose levels did not correlate with insulin levels as expected (Rand et al. 2002). They ascribed this stress induced hyperglycaemia to processes of gluconeogenesis needed for the fight or flight response (Rand et al. 2002). However, the above mentioned effects on glucose concentrations were reported in unsedated cats whereas the lions sampled in the current study were all sedated before handling, possibly indicating a bigger role of the anaesthetic drugs in the observed glucose and insulin responses.

It should however be noted that the age and gender based differences observed in the wild lion population to some degree are in line with what could be expected. Higher glucose concentrations in the wild juvenile lions were accompanied by higher insulin concentrations when compared to wild adult lions. This was also the case for wild adult male lions compared to wild adult females.

It has previously been suggested that increased insulin concentrations in cats are linked to the amount of body fat (Backus et al. 2007). In the current study insulin concentrations and BMI did not correlate when all the adult probably infected and uninfected lions were grouped together (Figure 4.14). However, when data of the infected and uninfected lions were separated, a relative strong positive correlation was observed (Figure 4.8). The fact that the uninfected subset had a stronger positive correlation between insulin and BMI than the infected subset (Figure 4.8) might be an indication of antagonism of insulin production by glucocorticoids such as cortisol (Woods et al. 1998). In the current study higher cortisol concentrations were observed in the probably infected lions compared to uninfected lions (Chapter 3). This observation is not supported by strong evidence and could be the subject of further investigation. However, the exposed and unexposed lions showed similar correlations between BMI and insulin despite exposed lions having higher cortisol

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concentrations than unexposed lions. This could either mean that the weaker correlation of the infected subset is not due to the effects of cortisol or that the selection criteria used to identify probably infected and uninfected lions were successful and thereby allowing for the effect of cortisol inhibition on insulin to be seen.

Insulin has also been reported to possibly affect leptin production (Woods et al. 1998). In the current study no correlations were observed between insulin and leptin concentrations. While this could be an indication that in lions the association of insulin and leptin are not as strong as in humans, it is also possible that the overlying affect of the drug Medetomidine on insulin production might be masking this link. Alternatively, it is also possible that either or both assays used to measure insulin or leptin are not giving true values thereby masking possible interactions.

Since body fat mass is a major confounding variable for leptin concentrations (Meier & Gressner, 2004), leptin concentrations in light of BMI were investigated. Except for lions from the infected subset, leptin concentrations in the current study did not correlate with BMI. One hypothesis might be that *M. bovis* in the probably infected lions is leading to weight loss and that this, together with pro-inflammatory cytokine inhibition of leptin in these lions is resulting in observable decreased leptin at lower BMI scores when these two markers are plotted against each other.

As part of the demographic study in the KNP each lion was given a body condition score on an ascending scale of 1 to 5 by the attending veterinarian on site. The probably infected KNP lions had scores ranging from 2.5 to 5 (mean ± SD, 3.53 ± 0.7518) with the highest juvenile score of 3 and the lowest adult score of 3. The two juvenile KNP lions classified as probably uninfected had body condition scores of respectively 3 and 4. Given the small probably uninfected KNP lion sample and lack of a similar scoring system in captive lions in the current study, it is not possible to draw relevant conclusions.

Previous reports of ghrelin concentrations in domestic cats showed that ghrelin concentrations decreased with increased body fat. In the current study ghrelin concentrations did not correlate with BMI in any of the wild/exposed, captive/unexposed, infected, or uninfected groupings. Despite this, linear regressions showed the slight general trend that could be considered normal for the exposed, infected and uninfected lions. The unexposed lions, however, showed a slight abnormal trend with increased ghrelin associated with higher BMI scores. Whether this is indicative of abnormal energy metabolism in captive/unexposed lions, or that the ghrelin RIA is insufficient for ghrelin analyses in lion blood, remains unclear.

Further attempts were made to identify possible interactions between the markers assayed in the current study. This was done by looking at the data of all the lions, just the adult lions, the probably infected and uninfected lions and the adults of the probably infected

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and uninfected subsets. Scatterplot matrix and correlation plots showed some positive or negative associations between some of the markers, however, none of the markers correlated (Spearman's, $r < 0.5$). BMI, leptin and glucose featured strongly in the first principle components (PC1) of the PCA's done while insulin and ghrelin featured more in the second principle component (PC2). However, when the exposure or infection status was superimposed over the PCA score and correlation plots no definitive groupings were observed. There were some indications that the exposed and infected lions associated more with higher insulin concentrations while the uninfected and unexposed lions associated more with the variation in leptin and glucose concentrations and BMI scores. Distance-based cluster analyses with all of the markers as well as with a selection of the markers that featured more in the previous statistical analyses were not able to correctly group the animals according to exposure or infection status. All of the above might therefore be an indication that the energy metabolism markers measured in this study are not suitable for use in discriminating between infected or uninfected lions. However, the likelihood that the assays used for the markers were inadequate also exists and the above conclusion is potentially premature.

4.5. Conclusion

In the light of the aims presented in section 4.1.6, the following conclusions and suggestions can be made: A major confounding factor in the current study was the use of human or canine assays to analyse the different markers of energy metabolism. Therefore, many of the observed differences or lack of correlations might not be due to the effect of *M. bovis* on energy metabolism but may be due to the inadequacy of the assays used. However, since most of the differences between age classes and genders were observed in the wild lions and not the captive lions, it might also be an indication that some form of altered energy metabolism is present in either the captive or wild population.

While it is not possible to draw definitive conclusions about the interactions of *M. bovis* infections on lion energy metabolism in the current study, there are some indications that this line of study should be the subject of further attention. Some aspects that will need to be kept in mind are the following:

- Lion specific assays are possibly needed for certain energy biomarkers.
- For energy metabolism marker investigations, the drugs used and sampling protocols need to be standardised as much as possible. This becomes more apparent when doing comparative studies between wild and captive lions.

- The possible effect of anaesthetic drugs on the markers needs to be kept in mind. Additional factors such as possible stress hyperglycaemia in restrained animals might also play a role.

- More value would be gained from identifying metabolic markers that give an idea of the state of energy homeostasis over an extended time period rather than trying to make sense of single time point measurements that have the ability to fluctuate rapidly. This becomes more important when working with wild lions for which one does not have control over feeding behaviour before the time of sampling.

- Despite %HbA1c not yielding worthwhile results in the current study, it might still be worthwhile to further investigate and develop the use of this marker to gain insights into lion glucose metabolism as it will likely not be affected by the use of drugs such as Medetomidine (a drug regularly used to sedate lions for experimental work and biological sampling), and is likely more resilient to changes in glucose concentrations due to meals shortly before sedation of the animal. While the current study made use of a human HbA1c assay routinely employed at an established laboratory, thereby reducing costs, there are more costly options of feline or canine HbA1c ELISAs that might be worth investigating.

- There is a need for baseline studies of the major energy metabolism markers in lions. While domestic cat data is available for some of these markers it is not possible to sedate and sample lions with the same protocols used in domestic cats. Interspecies differences might be considerable.

- Age and gender of lions need to be considered when designing future studies in order to avoid limitations in statistical analyses due to small sample sizes.

- Once assays sensitive to energy metabolism markers of lions have been developed it would be ideal to experimentally infect lions with *M. bovis* and to subsequently quantify the levels of said markers along the progress of infection and disease over an extended time.

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Chapter 5

Reproductive endocrinology of lions: Comparative analyses and novel interrogation protocols.

5.1 Introduction

Reproduction is one of the fundamental processes of life. Being able to successfully reproduce ultimately contributes to the survival and evolution of a species. Hormones play an essential role in reproduction and understanding the factors that influence endocrine function is key to maximising reproductive success of breeding programmes (Brown, 2006). This knowledge becomes important in the management and conservation of species threatened with extinction. One such species is the lion (*Panthera leo*). Lions have suffered more than a 30% population reduction in the past two decades and have subsequently been classified as vulnerable under the IUCN Red List of Threatened Species (Nowell et al. 2012).

In addition to the human threat faced by lions, other factors such as disease can also play a role in the decline of lion populations (Nowell et al. 2012). Consensus has not been reached about the possible negative effects of *M. bovis* infections and/or disease on lion population dynamics (Viljoen et al. 2015). While there are some indications that lion TB is affecting their ability to survive in their natural environment (Viljoen et al. 2015), no studies have been conducted with regards to the lions' neuro-endocrine reproductive ability in the context of *M. bovis* infections/disease.

A cascade of systems make up the reproductive system and normal functioning is regulated and maintained through various hormonal feedback loops as well as additional external inhibitors and stimulants. Simplified, the normal reproductive cascade consists of the kisspeptin (KP) system that through the production of kisspeptin peptides stimulates release of gonadotropin-releasing hormone (GnRH) which in turn stimulates secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Lehman et al. 2010; Pinilla et al. 2012; Roseweir & Millar, 2009). LH and FSH stimulate the production of gametes and the reproductive hormones such as testosterone and oestradiol. Kisspeptins are a family of neuro-peptides cleaved from an initial 145 amino acid peptide product (Kp-145) encoded by the *KISS1* gene. Kp-145 is cleaved into the shorter peptides Kp-54, Kp-14, Kp-13, and Kp-10 that all have a common C-terminal RF-amide and are ligands for the G-protein-coupled receptor KISS1R (a.k.a. GPR54) (Albers-Wolthers et al. 2014; George et al. 2011). The identification that inactivating mutations in human and mouse kisspeptin receptor (GPR54) led to a failure to progress through puberty demonstrated that the kisspeptins and GPR54 play a key neuro-endocrine role as gatekeepers of reproduction and that they are essential for the initiation of puberty and maintenance of adult reproduction (Lehman et al. 2010; Millar et al. 2010; Roseweir & Millar, 2009; Smith et al. 2006).

In recent years it has been shown that the KP system is sensitive to the levels of circulating hormones such as sex steroids, leptin and ghrelin which are indicative of, and affected by the state of energy homeostasis in an organism. In line with this, it has also been established that the KP system can be affected by various stress and inflammatory factors (Millar et al. 2010; Pinilla et al. 2012; Smith et al. 2006).

It is well documented that high levels of stress (Brown & Wildt, 1997; Pinilla et al. 2012; Viau, 2002) and conditions of immune/inflammatory challenges (Pinilla et al. 2012) can negatively affect the ability of an individual to successfully reproduce. The study by del Rey et al. (2007) found that in adult men testosterone concentrations decreased and oestradiol concentrations increased as the severity of *M. tuberculosis* disease increased. Their study did not investigate reproductive success but interpreted it in relation to the immune/inflammatory modulatory abilities of these hormones (del Rey et al. 2007).

In order to better understand the effects of stress on reproduction it is necessary to first understand how the reproductive system of the studied species functions normally. Through decades of study much has been learned about the reproductive biology and endocrine function of domestic cats. Some early studies have also described the hormone profiles of the oestrus cycle for some wild felid species. These early studies highlighted the diversity of reproductive endocrine characteristics that exists within the Felidae (Brown, 2006; Brown & Wildt, 1997).

One of the major themes that is highlighted in the review by Brown (2006) on the comparative endocrinology of domestic and wild felids is that although steroid metabolism may be conserved in the taxon, absolute production and/or excretion rates of steroids are more species specific (Brown, 2006). This is also highlighted by the species specific differences in oestrogen secretory patterns throughout gestation, species differences in seasonal reproductive patterns, and variation in ovulatory mechanisms within the taxon. In the latter case this variation can also occur within a species at individual levels (Brown, 2006).

Due to the diversity of reproductive endocrine mechanisms present in the Felidae it becomes difficult to make assumptions for a particular species reproductive abilities and functioning based on knowledge gained from another species within the same family. Some information is available for lions and will be summarised in the following sections.

The majority of knowledge on the reproductive hormonal characteristics of lions comes from studies conducted on females with more limited knowledge published on males. Investigators have made use of predominantly two methods (blood sampling or faecal sampling) to analyse pituitary gonadotropins and ovarian steroids (Brown et al. 1991; Brown et al. 1993; Schmidt et al. 1979; Schramm et al. 1994; Umapathy et al. 2007). These and

other studies provided a foundation for understanding the duration of the reproductive cycle, time of ovulation in relation to time of mating, and hormone secretion dynamics during pregnancy (Brown, 2006). Both methods have their own advantages depending on the question that needs to be addressed. The more invasive sampling of blood can give you real time hormonal profiles. Natural hormonal activity is dynamic and can fluctuate within minutes resulting in hormonal profiles that have peaks, midpoints and troughs. A single blood sample could therefore sample any part of the profile without the observer knowing which one (Brown & Wildt, 1997). Less invasive faecal sampling gives you an average/integrated value since metabolites are excreted over a period of hours allowing one to distinguish between normal secretory dynamics and genuine physiological responses (Brown, 2006; Brown & Wildt, 1997). By using both these methods in conjunction with behavioural observations valuable insights have been gained about the female lions' reproductive endocrinology and will be fist considered.

Despite initial reports of a preferred reproductive season for lions it is now accepted that lionesses do not have a seasonal reproductive cycle (Bertram, 1975; Brown, 2006; Brown & Wildt, 1997; Rudnai, 1973). Different environmental factors are believed to play important roles in the reproductive success of lions. Some of these factors include prey abundance (Bertram, 1975; Ferreira & Funston, 2010; Haas et al. 2005) and therefore nutritional status during early pregnancy (Rudnai, 1973), climate (Bertram, 1975), social factors and pride dynamics (Bertram, 1975; Haas et al. 2005; Rudnai, 1973), environmental stress (Tefera, 2003), genetic diversity (Munson et al. 1996; Wildt et al. 1987) and likely many other unidentified factors (Bertram, 1975).

Consensus has not been reached on whether lions are induced or spontaneous ovulators. Initially, lionesses were believed to be induced ovulators (Bertram, 1975), similar to domestic cats with the results from studies by Schramm et al. (1994) and Umapathy et al. (2007) in support of this. However, other studies suggested that this is not the case and that lionesses are polyoestrus with irregular cycles that vary in both length as well as time between periods (Bertram, 1975; Haas et al. 2005; Rudnai, 1973; Schmidt et al. 1979; Tefera, 2003). Bertram (1975) reported inter-oestrus intervals of between two weeks and several months with great variability within and between individuals. Oestrus periods also differed in intensity and length; most indicating they last between two and four days (Bertram, 1975).

A six month study investigating serum levels of oestradiol and progesterone of three female lions on a weekly basis found that the oestrus cycle of lionesses are more similar to that of the spontaneous ovulation of a dog than to that of the domestic cat, where coitus is necessary to stimulate ovulation (Schmidt et al. 1979). The oestrogen profile of the lion

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followed a similar pattern as that of other mammalian species. Baseline oestradiol levels were extremely low and only surged to a peak value in the follicular phase of the oestrus cycle after which they rapidly declined back to baseline levels. In the first two months of gestation the oestradiol levels remained stable at basal levels (Schmidt et al. 1979). During their study period, a total of nine oestradiol surges suggestive of oestrus (above 14 pg/ml) were recorded in the three females with a range of 19 to 108 pg/ml (Schmidt et al. 1979). Baseline oestradiol was usually extremely low at the limits of detection of the assay. Peak intervals varied between and within animals and ranged from three to eight weeks (Schmidt et al. 1979). Schmidt et al. (1979) however, mentioned that the weekly sampling protocol probably missed true peak values.

One of the above mentioned females fell pregnant during the study. She experienced an oestradiol surge of 19 pg/ml one week before mating. The morning after mating her oestradiol levels were non-detectable (Schmidt et al. 1979). After six weeks of pregnancy the oestradiol levels rose slightly to 11 pg/ml, dropped back to non-detectable the following week and stayed at < 5 pg/ml for rest of the time (Schmidt et al. 1979). Her progesterone concentrations rose from 1.6 ng/ml the week before mating to 49 ng/ml within 12 hours of mating and remained elevated (18-144 ng/ml) for the 2 months period of the pregnancy that she was sampled but was declining gradually from the peak at about the 4th week (Schmidt et al. 1979).

Progesterone profiles for non-pregnant females were unusual both in levels and patterns of progesterone secretion (Schmidt et al. 1979). Rises of progesterone after seven of the nine oestradiol surges suggest that ovulation had occurred. This, together with the fact that they had no contact with a male for six of these oestradiol surges suggested that lions are not contact/induced ovulators (Schmidt et al. 1979). Of the two cycles that did not result in elevated progesterone the one was similar to that seen in a non-mated domestic cat while the other was considered to be abnormal. Schmidt et al. (1979) mentioned progesterone levels reported in a previous study of < 10 ng/ml for non pregnant females and 50-100 ng/ml in lions considered to be pseudopregnant (Schmidt et al. 1979).

Schmidt et al. (1979) mentioned that progesterone levels in lions were much higher than in cats or dogs. The luteal phase lasted from 14 to 52 days in non-pregnant lions compared to 36 days in the cat and 80 days in the dog (Schmidt et al. 1979).

Valuable insights about the female oestrogen cycle were gained from behavioural observations and accompanying blood or faecal samples. However, due to the extended nature of collecting these samples and additional logistical difficulties, these methods are not ideal to describe in detail the functioning of an individual lions' reproductive neuro-endocrine system. This becomes important when one wants to establish if or how certain external

factors can influence a lions reproductive abilities. In this regard Brown et al. (1993) suggested using a GnRH challenge to assess pituitary function rather than just taking blood samples in anticipation of detecting a natural gonadotropin pulse.

Brown et al. (1991; 1993) were faced with the question of whether lack of genetic diversity was affecting reproductive capabilities of lions in the Ngorongoro crater in Tanzania. They conducted a GnRH challenge on both male (Brown et al. 1991) and female (Brown et al. 1993) lions. A summary of some of their findings are described below.

5.1.1 GnRH challenge of female reproductive system

The study by Brown et al. (1993) compared the responses to a GnRH challenge of female lions from two populations. The outbred control population originated from the Serengeti plains while the inbred population originated from the Ngorongoro crater. These populations were subdivided into treatment groups of animals receiving only saline injections (controls) and animals receiving the GnRH challenge. Additionally, to establish if an acute stress event might affect gonadotrophin secretion Brown et al. (1993) also had a treatment group subjected to an acute adrenocorticotrophin (ACTH)-induced increase in cortisol levels.

In the control animals basal levels of follicle stimulating hormone (FSH) stayed relatively stable throughout the procedure while pulses of luteinizing hormone (LH) secretion was observed at one to three pulses per four hours in half of the sampled females. Basal FSH levels were 34.7 ± 2.1 ng/ml for Serengeti and 27.9 ± 9.3 ng/ml for crater lions. Basal serum LH levels were 2.5 ± 0.4 ng/ml for Serengeti and 3.2 ± 0.8 ng/ml for crater lions.

In GnRH stimulated animals, LH concentrations peaked at 10-15 minutes after GnRH administration. The rise in FSH concentrations after GnRH stimulation was not as pronounced as the rise in LH concentrations (Brown et al. 1993) which is characteristic of mammals. Brown et al. (1993) did not give the precise values for LH levels after GnRH stimulation. The increased LH levels fell within the range of 15 to 29 ng/ml. FSH levels increased from an overall average of 31.7 ± 2.3 ng/ml to 45.7 ± 3.6 ng/ml after GnRH stimulation. Both LH and FSH responses to GnRH stimulation was unaffected by the higher levels of cortisol due to ACTH stimulation (Brown et al. 1993).

Progesterone and oestradiol secretion were not affected by GnRH or ACTH and did not differ between the study populations. Pregnant females had higher progesterone levels than non-pregnant females, but these levels were not as high as reported for a captive pregnant female by Schmidt et al. (1979) (Brown et al. 1993). Progesterone levels fluctuated during sampling period in all but two females, regardless of pregnancy, location, ACTH treatment or GnRH treatment. Although there were fluctuations of progesterone levels in non-pregnant females, it never reached as high as that of the pregnant females. The progesterone concentrations in pregnant females did not differ between study populations.

Serum oestradiol levels (ranged between 7.9 and 51.4 pg/ml) were not influenced by hormonal treatment or pregnancy and were stable during the sampling period. The overall mean oestradiol levels did not differ significantly between the populations (Brown et al. 1993).

5.1.2 GnRH challenge of male reproductive system.

The study by Brown et al. (1991) identified three stages of sexual development in male lions namely: 1) A prepubertal period with increased pituitary sensitivity to GnRH (at a dose of 1µg/kg) coupled with low testicular sensitivity to endogenously released LH; 2) A transitional post-pubertal period with pituitary sensitivity reduced to adult levels but still diminished testicular responsiveness; 3) An adult period of maximal testicular responsiveness but same/similar pituitary sensitivity as in the post-pubertal period (Brown et al. 1991).

As with the females, males from the Serengeti plains were compared to males from the Ngorongoro crater. The males were subjected to only one of two treatments namely the control treatment (only saline injected) or the GnRH challenge. Males were not subjected to an acute ACTH-induced increased cortisol treatment (Brown et al. 1991). Similar to what was seen in the females, no significant differences were observed between Serengeti plains lions and Ngorongoro crater lions with regards to LH, FSH and testosterone responses to the GnRH challenge. Age related differences were observed in LH and testosterone responses (Brown et al. 1991).

For the control males the basal serum LH and FSH concentrations were stable over time and unaffected by age or location. However, pulsatile LH secretions of 1-3 pulses/ 4 hour sampling period were observed in 2 of 5 adults, 2 of 4 young adults, and all of the prepubertal males. The LH pulses were on average 3.6 ± 0.7 ng/ml above baseline concentrations. No FSH pulses were observed (Brown et al. 1991) which is typical of many species studied.

The LH concentrations peaked within 15-25 minutes after GnRH administration with the LH peak of prepubertal males approximately 35% greater than that of adult and young adult males which were similar to each other. GnRH-induced LH response for males (Brown et al. 1991) were similar to that described in females (Brown et al. 1993). Brown et al. (1993) mentioned that this differed from what was previously observed in other feline species such as cheetah, leopard and tiger where the LH release after GnRH stimulation were several fold higher in males than in females.

Baseline FSH concentrations averaged 29.3 ± 0.2 ng/ml and increased to 49.8 ± 0.4 ng/ml after GnRH stimulation. The magnitude of FSH response to GnRH stimulation was not age dependent and was similar to that observed in other felids (Brown et al. 1991). However,

testicular FSH receptor concentrations were higher in prepubertal males than in adult males. Brown et al. (1991) suggested that this might be linked to higher testicular FSH receptor concentrations that precede the initiation of spermatogenesis, similar to what is observed in the rat, bull, and boar. In the case of lions, onset of spermatogenesis normally occurs roughly at two years of age (Brown et al. 1991).

In approximately 80% of the adult and young adult males the basal testosterone concentrations decreased between 20 and 50% over time until the GnRH was administered. This decline in basal testosterone was similar to what was seen in the leopard. It was, however, not seen in tiger, puma, or cheetah (Brown et al. 1991). Therefore, the effect of handling and/or anaesthesia may be species specific in felids (Brown et al. 1991). The greatest testosterone response to GnRH stimulation was observed in adult lions with the lowest response observed in prebubertal males (Brown et al. 1991).

GnRH induced LH production was similar in both young adult and adult males but young adult males did not produce the marked rise in testosterone measured in the adult males (Brown et al. 1991). While in most other species maximal testosterone secretion is achieved at the time of puberty or shortly thereafter, this study showed indications that for lions there appears to be a more gradual increase in testicular sensitivity to LH with maximal testosterone secretion not occurring until approximately 1-2 years after puberty (Brown et al. 1991). The age-related increases in testosterone production were positively associated with increased LH receptors in testes, rather than to increases in circulating LH concentrations (Brown et al. 1991). Brown et al. (1991) suggested that the increased pituitary sensitivity in the prepubertal males could be ascribed to increased pituitary content of LH or greater concentrations of GnRH receptors, as was previously described for rats. The reduced pituitary sensitivity in adult and young adult males could possibly be explained by the negative-feedback control of higher circulating baseline levels of testosterone. Testosterone is known to decrease pituitary GnRH receptors and attenuates pituitary responsiveness to GnRH (Brown et al. 1991).

None of the above mentioned studies interrogated the role of the KP system in lions. Because KP neurons seem to be integrative sensors that respond to changes in numerous internal and external factors, which include nutrient and fat status, stress hormones, and reproductive steroids, a link is provided between all these factors and gonadotropins (Millar et al. 2010; Pinilla et al. 2012).

5.1.3 KP system provides a link between the hypothalamic-pituitary-gonadal (HPG) axis and energy metabolism

Pioneering work on the development of kisspeptin antagonist demonstrated the essential role for KP in GnRH neuron firing, GnRH pulsatile secretion, negative feedback by gonadal steroids, the onset of puberty, and the ovulatory LH surge. These studies establish that kisspeptin antagonists are powerful investigative tools and set the scene for more extensive physiological and pathophysiological studies as well as therapeutic intervention (Millar et al. 2010).

It is well recognised that there is an interrelationship between the reproductive system and energy metabolism. In this regard, decreased amounts of the hormone leptin due to a negative energy balance have been associated with inhibition of GnRH (Tena-Sempere, 2010; True et al. 2011). Because GnRH neurons do not express leptin receptors (True et al. 2011; Young et al. 2013), upstream regulation through means of the KP system has been put forward (Gianetti & Seminara, 2008; True et al. 2011). Substantial data supports a central role for the KP/GPR54 system in mediating the effects of metabolism on the reproductive system. For example, *Kiss1* expression in the ARC (arcuate nucleus) was reduced in castrated leptin-deficient ob/ob mice, but increased with the administration of leptin (Millar et al. 2010; Pita et al. 2011). However, the study by True et al. (2011) found that restoring leptin concentrations to normal basal levels did not result in the restoration of normal *Kiss1* and subsequently LH production. The therapeutic effect of leptin on the KP system was only seen when pharmaceutical levels of leptin were administered (True et al. 2011). They concluded that reduction in leptin levels might not be the critical signal for the inhibition of *Kiss1* and LH during a negative energy balance but that it may play a permissive role (True et al. 2011). Further examples of the interconnectedness of energy metabolism and reproduction through the KP system include: links of the KP system with decreased LH secretion in rats with uncontrolled diabetes; interactions with glucose induced insulin production; and seasonal breeding cycles through effects of photoperiod on *Kiss1* expression (Gianetti & Seminara, 2008; Roseweir & Millar, 2009).

In the view of the role of the KP system in mediating the effects of inflammation, stress and energy metabolism on the reproductive endocrine system a comparison of HPG axis responses to KP in healthy and *M. bovis* infected and/or diseased lions was proposed. The aim of this part of the study was to interrogate the lion reproductive endocrine system by way of the KP system. The hypothesis was that: TB will compromise reproductive capacity even if it does not cause major morbidity or mortality. It is further hypothesised that this compromised capacity will be due to the known inhibition of KP gene expression by inflammation and impaired nutritional conditions which may result from TB, and the downstream reduction in GnRH, gonadotropin and gonadal sex hormone production.

5.2 Materials and methods

5.2.1 Study animals

For the purpose of the KP challenge of the HPG axis, lions that were believed to be unexposed to *M. bovis* were selected. These lions were housed at the Pretoria Zoo and the Rietvlei Nature reserve facilities of National Zoological Gardens of South Africa (NZG) and at the Ukutula Lion Centre (Ukutula) in South Africa. The experiment was conducted on both males and females in different age classes. Section 5.2.2 describes the experimental procedure.

Additionally, blood samples collected from the KNP lions used in this thesis were assayed for testosterone (males), progesterone (females), and oestradiol (females). Blood was collected in EDTA (lavender top) tubes and kept on ice until processing at the laboratory the same night. On arrival at the laboratory the EDTA tube was centrifuged at 3000 rpm for 10 min. The plasma was then divided into approximately 500 µl aliquots. All the aliquots were then frozen at -20 ºC until further analyses. Note: Although the intention was to compare the reproductive hormone responses in KNP lions with and without TB, the early termination of the study by SANParks did not allow for KP stimulation studies in the wild lions.

5.2.2 Experimental procedure and sampling

Ethical clearance and permission to conduct the experiment were obtained from all participating institutions as referred to in Chapter 2. Qualified veterinarians were in charge of administrating the necessary drugs and looking after the welfare of the animals.

The lion selected for the procedure were separated from the rest of the pride into a smaller management camp. The NZG lions were immobilized with a combination of Medetomidine, Midazolam, and Butorphanol via remote injection dart (Daninject). Ukutula lions were immobilised with either a combinations of Medetomidine and Zoletil or Medetomidine and Ketamine. See Chapter 2 section 2.2.2 to 2.2.4 for dosage concentrations of the anaesthetising drugs. Once the lion was non-responsive to stimuli it was moved to the working station. It was necessary to maintain the lions under a light surgical anaesthesia throughout the experimental procedure. At the NZG lions were intubated and maintained on Isoflurane in oxygen. Ukutula lions received follow up injections of a combination of Medetomidine and Ketamine as needed.

Before commencement of the experiment each lion was weighed and body measurements taken for body mass index (BMI) studies (BMI results discussed in Chapter 5). A 14 gauge intravenous catheter was placed in one of the medial saphenous veins for the intermittent collection of samples. The catheter was flushed with heparin in saline after

each sample collection. Initial blood samples were taken for assessment of the metabolic and immune/inflammatory markers as described in the previous chapters.

For the experimental procedure blood samples were collected at 10 minute intervals into EDTA tubes. Different experimental protocols were implemented whereby either Kisspeptin-10 (Kp-10) alone, GnRH alone, or both were injected and samples taken over an extended time period. The basic protocol consisted of 60 minutes of baseline sampling after which a dose of 1 µg/kg Kp-10 was injected intra venous (IV). This was followed by 90 minutes of sampling after which a dose of 1 µg/kg GnRH was injected IV followed by a further 30 minutes of sampling. The GnRH dose selected was in line with the 1 µg/kg dose previously used on lions by Brown et al. (1991; 1993). Since no previous studies were done using Kp-10 to interrogate lion neuro-endocrine function, guidance was taken from a human study (George et al. 2011) that found a 1 µg/kg dose of Kp-10 was the minimum required dose to get a maximal LH response. One lion served as a control animal and no Kp-10 or GnRH was injected for the duration of the 180 minutes of sampling. Since part of this study was aimed at development of a protocol to investigate lion reproductive neuro-endocrine function, some alterations were made as the study progressed. These alterations will be discussed in the results and discussion sections of this chapter.

All blood samples were kept on ice (not frozen) until the end of the procedure after which they were centrifuged for 10 minutes at 6000 rpm. The blood plasma was then removed and aliquoted into 1.5 ml vials and frozen at -20 ºC until the time of further analyses.

At completion of the experiment the lions were moved back to the management camp and the anaesthesia was reversed. Each lion were observed until the veterinarian was satisfied that it had completely recovered before allowing it to rejoin the pride.

5.2.3 Assays

<u>5.2.3.1 Testosterone</u>

Blood samples of the KNP males and three NZG lions were assayed with a total testosterone RIA (Coat-a-count® Total Testosterone, Siemens Healthcare Diagnostics, USA). This assay was discontinued by the manufacturer halfway through the study period – that stretched over two years - resulting in only some samples to be analysed with this assay. This RIA has not been validated for lions, however, a parallelism test and non-linear curve fit analyses (F-test) were conducted. A parallelism test compares a sample dilution curve against the standard curve of the assay. If the slopes of the sample dilution curve and the standard curve do not differ significantly one can assume that the substance measured in the lion plasma has similar immunological activity as the testosterone standard and is therefore likely representative of circulating lion testosterone.

One sample with a high testosterone concentration was double diluted five times using the assay buffer. The resultant dilution curve was compared to the RIA standard curve (see Figure 5.1). The parallelism test showed that the sample dilution curve and the standard curve did not differ significantly for the standard testosterone range of 3.5 to 55 nmol/L. It can, therefore, be assumed that other constituents in the lion plasma does not interfere significantly with the testosterone assay within the above mentioned concentration range. Therefore, these results are likely representative of the testosterone concentrations in lion plasma.
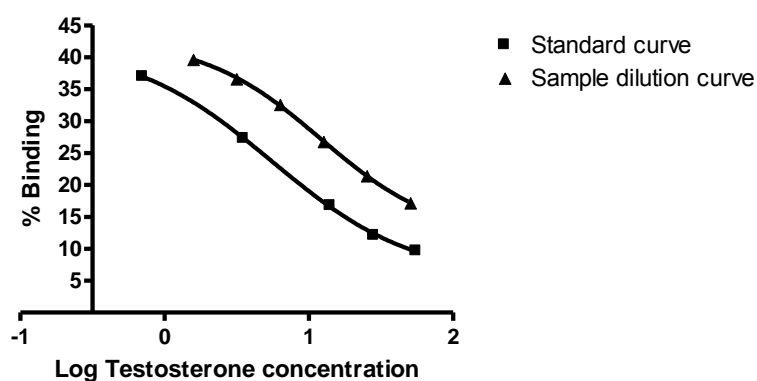
Figure 5.1: Graph showing the parallelism results for the Siemens Testosterone RIA kit

Total circulating testosterone of the remainder of the NZG and Ukutula males were measured with the IBL Testosterone RIA (Testosterone RIA (CT), IBL International, Germany). The assay was done in accordance to the manufacturers guidelines. As with the discontinued assay (Siemens RIA), a parallelism test and non-linear curve fit analyses (F-test) were done for this RIA (see Figure 5.2). The standard curve and the dilution curve did not differ significantly for the standard testosterone range of six to 385 ng/dL. Therefore, although not reporting the actual testosterone concentrations results between six and 385 ng/dL are likely representative of the total testosterone concentrations in lion plasma.

Samples of two of the three NZG lions that were assayed with the Siemens RIA were again assayed with the IBL RIA (See Figure 5.9). Since the Siemens RIA reported testosterone concentrations in nmol/L, an unit conversion to ng/dL (concentration unit used by IBL RIA) was done and it was established that the testosterone concentrations measured with the IBL RIA was on average 33.5% of that measured with the Siemens RIA with similar testosterone response curves to Kp-10 stimulation generated by both the Siemens and IBL RIA's. Unfortunately, due to a limited volume of blood sample, all of the wild lion samples could not be re-run with the replacement IBL RIA.
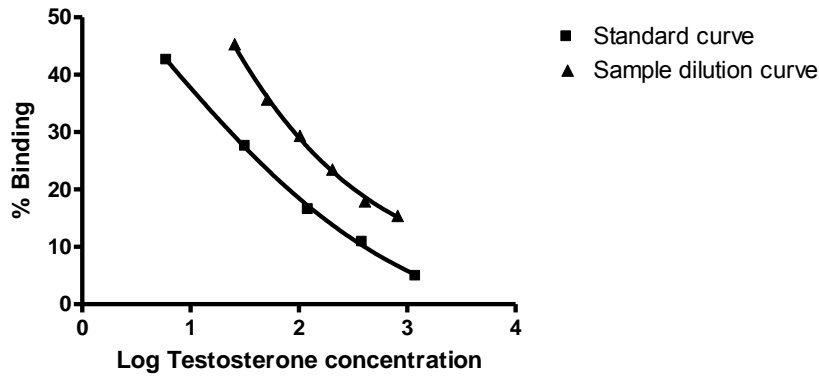
Figure 5.2: Graph showing the parallelism results for the IBL Testosterone RIA kit

5.2.3.2 Luteinizing Hormone (LH)

Previous studies showed that LH secretion in both male and female lions are sensitive to stimulation by GnRH (Brown et al. 1991; Brown et al. 1993). We attempted to make use of a multi species LH ELISA (LH ELISA (Serum), Demeditec Diagnostics GmbH, Germany), but was unable to measure LH in the lion plasma samples. We are currently attempting to develop an ELISA capable of measuring lion LH, but no results were available at the time that the thesis needed to be submitted.

5.2.3.3 Progesterone

Blood samples from all the KNP females and seven NZG females were assayed with a progesterone RIA (Coat-a-count® Progesterone, Siemens Healthcare Diagnostics, USA). The assay was done in accordance with the manufacturers guidelines. This assay was not validated for lion blood. One blood sample that yielded the highest progesterone concentration was used to conduct a parallelism test and non-linear curve fit analyses (F-test). The sample was double diluted (5 times) and a dilution curve generated. The dilution and the standard curves (Figure 5.3) did not differ significantly for progesterone concentrations below 31.8 nmol/L. It can therefore be assumed that constituents in lion plasma did not interfere with progesterone measurement at concentrations below 31.8 nmol/L and results below this concentration are likely representative of progesterone concentrations in lion plasma.

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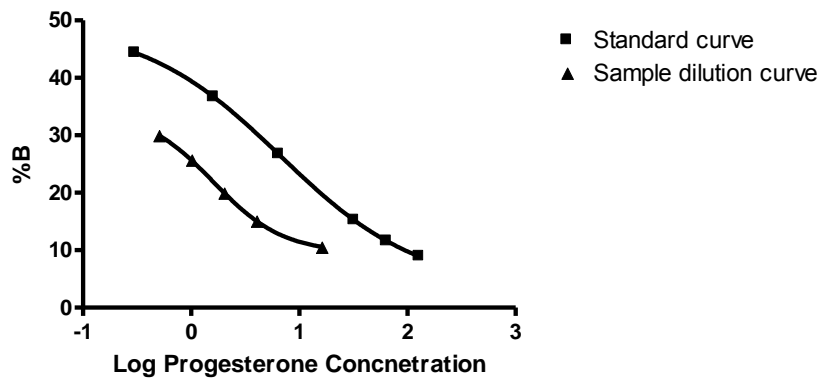
Figure 5.3: Graph showing the parallelism results for the Progesterone RIA kit.

5.2.3.4 Oestradiol

Blood samples from all the KNP females and three NZG females were assayed for oestradiol concentrations by means of an oestradiol RIA (Coat-a-Count® Oestradiol, Siemens Healthcare Diagnostics, USA). The assay was conducted in accordance with the manufacturer guidelines. This assay has not been validated for lions. All oestradiol concentrations were below the limit of detection of the assay. Due to results described by (Brown et al. 1993) whereby serum oestradiol concentrations were not influenced by GnRH stimulation in the experimental sampling time window (240 minutes), no further attempts were made to obtain oestradiol concentrations for females in the current study.

5.2.4 Data analysis and statistics

Small sample numbers prevented proper statistical comparisons. Visualisations of response curves and basic statistics were done with GraphPad Prism Version 4 for Windows (GraphPad Software, Inc., CA, USA). Area under the curve (AUC) of the testosterone response profiles were calculated using the trapezoidal method. Because the time windows before and after administration of either Kp-10 or GnRH differed the AUC's could not be compared as is. For comparison purposes the AUC's were calculated by only considering the time windows of equal length. For example, if the total sampling time preceding Kp-10 administration was 60 minutes and the sampling time after Kp-10 administration until GnRH administration was 90 minutes, then the AUC's were calculated for only 60 min before and after Kp-10 administration.

In some instances age class comparisons were done. Age related data inspections were done using one of two age classification systems. The first was based on age classes identified for male lions in Brown et al. (1991) with classes defined in accordance with reproductive development as adult (> 5 years), young adult (2-5 years), and prepubertal (< 2 years). The second was based on the age classification used by the KNP with classes defined as adult (> 4 years), sub-adult (2-4 years), and juvenile (< 2years).

5.3 Results

5.3.1 Single time point measurements

As mentioned before, single time point blood samples were collected from KNP lions. As part of the kisspeptin study, blood samples were collected from the captive lions at 10 minute intervals throughout the experimental procedure. Therefore, in order to compare hormonal concentrations of captive (*M. bovis* unexposed) and wild (*M. bovis* exposed) lions, the hormonal results obtained from the first baseline blood sample (Sample at time = T0) of the captive lions were used.

5.3.1.1 Testosterone

Some of the age classes had too few animals for meaningful comparative statistics. A summary of the testosterone concentrations can be seen in Table 5.1. Table 5.2 gives the demographic information of all the male lions. Since the parameters of the Brown age classification system differed from the KNP system it is possible that a lion considered an adult with the KNP system could be classified as young-adult with the Brown system. For the wild/KNP lions the structure of the age classes did not change between the different classification systems. The majority of wild males were grouped in the pre-pubertal or juvenile age class. More captive lions were classified as adult with the KNP system than with the Brown system.

Within the wild lions (Figure 5.4; Table 5.1), the mean testosterone concentration of adult lions was greater than that of the young-adult/sub-adult and the pre-pubertal/juvenile lions. Juvenile/pre-pubertal wild lions had the lowest testosterone concentrations.

The captive lions (Figure 5.5; Table 5.1) only consisted of lions that were considered to be adult or young- /sub-adult. According to the Brown age classification adult lions had lower mean testosterone concentrations than young-adult lions (as measured by IBL RIA). One captive adult (NZG10) that only had a Siemens RIA results was initially excluded. When the calculated testosterone concentration of NZG10 was included, the mean testosterone concentration of adult lions was 73.8 (31.2) (Mean (SD)) and therefore similar to that of young-adults. Grouping the captive lions according to the KNP age classification showed that the mean testosterone concentration of adult lions (with and without NZG10) was approximately 2.5 times greater than that of sub-adult lions.

In order to compare between wild and captive lions, the concentrations in wild lions obtained with the Siemens RIA were used to calculate a theoretical IBL RIA result (see Tables 5.1 and 5.2 for adjustments of Siemens RIA values to IBL RIA values). Captive adult lions had higher testosterone concentrations than wild adult lions. Similarly captive young-/sub-adult lions had higher testosterone concentrations than the wild young-/sub-adult lions.

Table 5.1: Summary of testosterone concentrations for single time point blood samples of all the male lions. Data as mean ± SD.

Sample location	Age class	Testosterone (ng/dL)		
		Siemens	IBL	Siemens adjusted to IBL levels*
KNP/Wild	Adult (n=3)	106.6 ± 60.4	-	35.7 ± 20.2
	Young-adult/ Sub-adult (n = 1)	46.1	-	15.5
	Pre-pubertal/ Juvenile (n = 10)	15.7 ± 12.1	-	5.3 ± 4.1
Captive[1]	Adult (Brown) (n = 2)	-	55.8 ± 2.2	-
	Adult (KNP) (n = 6)	-	91.4 ± 67.1	-
	Young-adult (Brown) (n = 8)	-	73.4 ± 64.8	-
	Sub-adult (KNP) (n = 4)	-	37.5 ± 12.3	-

* Testosterone concentrations calculated as 33.5% (see section 5.2.3.1) of Siemens RIA result in order to compare with IBL RIA results.

[1] One captive male for whom only a Siemens RIA result was available was not included. Its' Siemens RIA testosterone concentration was 327.7 ng/dL with the calculated IBL RIA concentration at 109.8 ng/dL. It was classified as adult with both the KNP and Brown age classification systems.

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Table 5.2: Demographic information and testosterone results of male lions sampled in this study. Lions highlighted in orange indicate individuals for whom there were two sampling events. (Infection status = probability of infection according to results described in Chapter 3; QFT *M. bovis* status = Classification according to the GEA results discussed in Chapter 3; FIV = Feline immunodeficiency virus diagnosis; Stat-Pak = TB Stat-Pak diagnosis; BAL = Bronchoalveolar lavage diagnostic results; Skin-test = intradermal tuberculin skin test diagnosis)

Lion #	Infection status	Age	*M. bovis* exposure	#Age class		QFT *M. bovis* status	FIV	Stat-Pak	BAL	Skin-test	Testosterone ng/dL		
				KNP	Brown						Siemens	IBL	Adjusted to IBL equivalent*
KNP03	Probably infected	3yr	*M. bovis* exposed	SA	JA	probable	Positive	Negative	Negative	Positive	46.1		15.4
KNP07	Probably infected	10-12mth	*M. bovis* exposed	JU	PP	suspect	Positive	Negative	Negative	Positive	23.6		7.9
KNP09	unknown	6mth	*M. bovis* exposed	JU	PP	probable	Positive	Negative	Negative	Negative	7.8		2.6
KNP15	Probably uninfected	18-24mth	*M. bovis* exposed	JU	PP	unlikely	Positive	Negative	Negative	Negative	46.1		15.4
KNP22	unknown	6-8mth	*M. bovis* exposed	JU	PP	unlikely	Positive	Negative	Negative	Positive	4.3		1.4
KNP22	unknown	12mth	*M. bovis* exposed	JU	PP						42.8		14.3
KNP24	unknown	4-6mth	*M. bovis* exposed	JU	PP		Positive	Negative	Negative	Positive	8.6		2.9
KNP25	Probably uninfected	7-8yr	*M. bovis* exposed	AD	AD	unlikely	Positive	Negative	Negative	Negative	41.5		13.9
KNP26	unknown	4-6mth	*M. bovis* exposed	JU	PP	suspect	Positive	Negative	Negative	Negative	17.3		5.8
KNP27	Probably infected	4-6mth	*M. bovis* exposed	JU	PP	probable	Positive	Negative	Negative	Positive	9.2		3.1
KNP29	Probably infected	5yr	*M. bovis* exposed	AD	AD	probable	Positive	Negative	Negative	Positive	160.7		53.8
KNP31	unknown	7-8yr	*M. bovis* exposed	AD	AD	unlikely	Positive	Negative	Negative		117.7		39.4
KNP36	unknown	8-10mth	*M. bovis* exposed	JU	PP	unlikely	Positive	Negative	Negative	Positive	9.7		3.2

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Lion #	Infection status	Age	*M. bovis* exposure	#Age class		QFT *M. bovis* status	FIV	Stat-Pak	BAL	Skin-test	Testosterone ng/dL		
				KNP	Brown						Siemens	IBL	Adjusted to IBL equivalent*
KNP38	Probably infected	10mth	*M. bovis* exposed	JU	PP	probable	Positive	Negative	Negative	Positive	16.4		5.5
NZG 04	Probably uninfected	3yr 1mth	*M. bovis* unexposed	SA	JA	unlikely	Negative	Negative	Negative	Negative	165.1	51.4	55.3
NZG 07	unknown	5yr	*M. bovis* unexposed	AD	AD	unlikely	Negative	Negative	Negative	Positive	208.0	57.3	69.7
NZG 10	Probably uninfected	7y10mth	*M. bovis* unexposed	AD	AD	suspect	Negative	Negative	Negative	Negative	327.7		109.8
U04	Probably uninfected	4yr10mth	*M. bovis* unexposed	AD	JA		Negative	Negative	Negative	Negative		212.2	
U05	Probably uninfected	4yr2mth	*M. bovis* unexposed	AD	JA		Negative	Negative	Negative	Negative		27.0	
U14 aka U05	Probably uninfected	4yr9mth	*M. bovis* unexposed	AD	JA							48.0	
U06	unknown	2yr7mth	*M. bovis* unexposed	SA	JA	probable	Negative	Negative	Negative	Negative		31.7	
U09	unknown	2yr11mth	*M. bovis* unexposed	SA	JA	unlikely	Negative	Negative	Negative	Positive		43.3	
U10	unknown	2y8mth	*M. bovis* unexposed	SA	JA	suspect	Negative	Negative	Negative	Positive		23.8	
U16	unknown	14yr8mth	*M. bovis* unexposed	AD	AD	suspect	Negative	Negative	Negative	Positive		54.2	
U20 aka U16	unknown	4yr10mth	*M. bovis* unexposed	AD	JA							251.3	
U17	unknown	4yr3mth	*M. bovis* unexposed	AD	JA	unlikely	Negative	Negative	Negative	Positive		122.3	
U18	unknown	4yr3mth	*M. bovis* unexposed	AD	JA	unlikely	Positive	Negative	NTM	Negative		75.2	
U21 aka U18	unknown	14yr8mth	*M. bovis* unexposed	AD	AD							8.1	

See next page for * and #

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* Testosterone concentrations calculated as 33.5% (see Section 5.2.3.1) of Siemens RIA result in order to compare with IBL RIA results.

[#] The two age classification systems used in this study were the Brown et al. (1991) system (Brown) and the system used by the Kruger National Park's (KNP) veterinarians (See Section 5.2.4). The Brown system classifies individuals as pre-pubertal (PP), young adult (JA), and adult (AD). The KNP system classifies individuals as juvenile (JU), sub-adult (SA), and adult (AD).
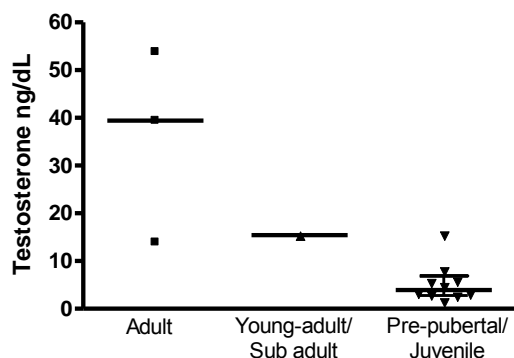
Figure 5.4: Testosterone concentrations of KNP males assayed with the Siemens RIA. Lines represent medians and interquartile ranges.
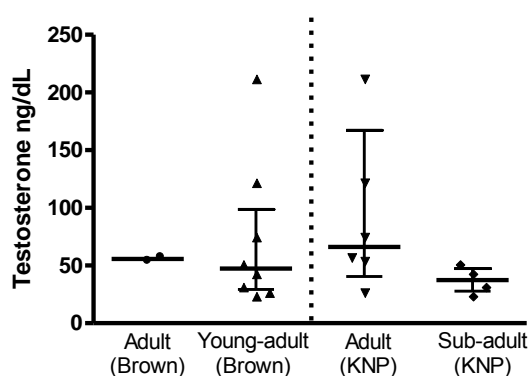
Figure 5.5: Testosterone concentrations of captive male lions assayed with the IBL RIA grouped in accordance to the Brown age classification (left of dotted line) and the KNP age classification (right of dotted line). Lines indicate median and interquartile ranges.

5.3.1.2 Progesterone

Since the Brown age classification system is based on male lion reproductive development, this classification system was not used on female lions. Blood samples from all the KNP female lions were analysed for progesterone. Blood samples from only seven captive female lions (originating from the NZG) were analysed. The preliminary results (as described below and in Section 5.3.2.2) together with the results described in Brown et al. (1993) lead me not to analyse the blood from the rest of the captive females.

In the wild lions, only three adult females had progesterone concentrations greater than the limit of detection of the assay. Of the seven captive females that were analysed for progesterone, one adult and both sub-adults had progesterone concentrations greater than the limit of detection. Because the results obtained for the six females mentioned above only indicated that their progesterone concentrations were > 127.2 nmol/L these animals could not be included in the comparative statistics. The progesterone concentrations of wild adults did not differ significantly from wild juveniles or from captive adults (Figure 5.6).

As part of the overall sampling procedure in the KNP, notes were made on body condition and state of pregnancy. The following observations were made for the three wild females with high progesterone concentrations:

- KNP05 – "Late pregnant or cubs on foot" and lactating
- KNP19 – "Possibly early pregnant" and not lactating
- KNP20 - "Possibly pregnant" and not lactating.

Of the captive females two were not cycling at the time of sampling and one did not have information on reproductive status. However, it was very unlikely that any of the captive females were pregnant as all of them were housed with males that had previously been vasectomised. It should be noted that two of the three captive females with high progesterone concentrations were housed together and in an enclosure directly next to two other females of which one was the third female with high progesterone concentrations.
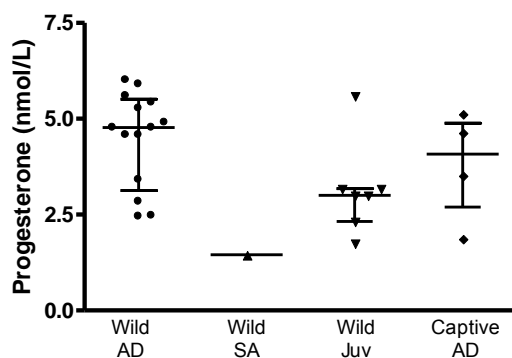
Figure 5.6: Scatter-dot plots of progesterone concentrations according to age classification for the wild/KNP lions and four of the seven captive lions. Progesterone concentrations above the maximal level of quantification were not plotted. (Solid lines indicate median and interquartile ranges; AD=adult; SA=Sub-adult; Juv=Juvenile)

5.3.2 Kisspeptin challenge

5.3.2.1 Testosterone

Introductory notes

Due to a limited number of males, some Ukutula males were used twice for different protocols (Ukutula lions highlighted in orange in Table 5.2). The least amount of time between two protocols on an individual lion was nine days which is in line with clinical trial practice when using human subjects more than once. A total of five males were subjected to the standard protocol of 60 minutes baseline sampling, IV injection of Kp-10 (1 µg/kg), 90 minutes of response sampling, IV injection of GnRH (1 µg/kg), and 30 more minutes of sampling. An attempt was made to reduce the time a lion is under sedation by reducing the total sampling time to 120 minutes and only injecting Kp-10 after 60 minutes of baseline sampling. One control male was baseline sampled for a total of

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180 minutes. Three males received only an IV GnRH injection after 60 minutes of baseline sampling with sampling continuing for another 120 minutes thereafter. Additionally, two males were subjected to an extended procedure with IV Kp-10 injection after 60 minutes of baseline sampling, 90 minutes of response sampling, injection of GnRH and then a further 60 minutes of sampling.

As mentioned before, two age classification systems could be used to inspect the testosterone responses to Kp-10 and GnRH stimulation. Depending on the classification system used some lions could either be classified as young adults (Brown classification) or as adults (KNP classification). This had an effect on the differences observed between the younger and older lions. For completeness purposes the results will be given considering both classifications, however, during the discussion focus will be given to results according to the KNP age classification.

General summary

A summary of the testosterone responses for each lion can be seen in Table 5.3. Except for the three males with the shortened protocol (Figure 5.15), one male with the standard protocol (Figure 5.10), and the control male (Figure 5.8), the first 70 minutes of sampling for the rest of the males showed a general decline in circulating testosterone likely due to the effects of the drugs used for sedation (See discussion under section 5.4.1.1). Although the control male showed an initial increase in testosterone for the first 30 minutes of baseline sampling, testosterone concentrations declined for the following 110 minutes after which it stayed constant until the end of sampling. Irrespective of the treatment, 71% of the lions had a 16 to 64% (mean ± SD, 43.94 ± 14.19%) decline in basal testosterone concentrations during the first 70 minutes of the sampling procedure (Figure 5.7). The mean half life of total testosterone in male lions from the current study was calculated to be ~50 minutes. The majority (n = 6/9, 66.7%) of males showed an increase in circulating testosterone within one hour of Kp-10 administration. The mean increase of testosterone concentrations 50 min after Kp-10 administration was 282.2 ± 120.4% of the basal testosterone concentration measured just before Kp-10 injection. The greatest testosterone response to the Kp-10 challenge was observed in a 13 year old male (U21). This is likely due to this male having the lowest reported basal testosterone levels for this study (Figure 5.14). We would anticipate that the response to Kp-10 would be greater if the basal levels are lower.
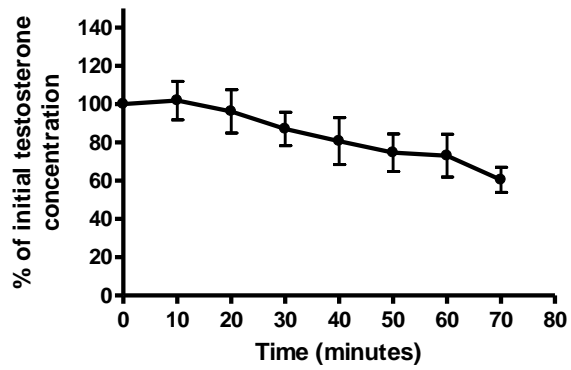
Figure 5.7: Mean testosterone concentrations as a percentage value of the testosterone concentration in the first blood sample of all the male lions that showed a decrease in basal testosterone concentrations during the first 70 minutes of sampling. Vertical T bars represent SEM.

None of the lions that received only a GnRH injection showed an increased testosterone response (Figure 5.16). Testosterone responses to GnRH administration were only observed in the two lions that were part of the extended protocol (Table 5.3, Figures 5.13 and 5.14). None of the standard protocol lions (Table 5.3) showed a response to GnRH.

Table 5.3: Summary of lions allocated to each treatment with their subsequent testosterone response. Super script letters indicate individual lions subjected to different treatments.

	Lion	Age (years)	Response to Kp-10	Response to GnRH	Initial Testosterone (ng/dL)	Lowest Testosterone (ng/dL)	Highest Testosterone (ng/dL)
Control	U14[a]	4.74	-	-	48	15.2	80.9
Standard protocol	NZG4	3	Y	N	51.4	19.1	92.1
	NZG7	5.17	Y	N	57.3	35.5	107.4
	NZG10*	7.83	N	N	109.8	84.5	152.9
	U9	2.89	Y	N	43.3	15.2	80.1
	U10	2.62	Y	N	23.8	15.2	31.7
Mean ± SD					57.12 ± 32.05	33.9 ± 29.5	92.84 ± 43.93
Extended protocol	U20[b]	4.95	Y	Y	251.3	133.7	274.1
	U21[c]	13.22	Y	Y	8.1	< LO	74.8
Mean ± SD					129.7 ± 172	133.7	174.5 ± 140.9
Shortened protocol	U4	4.18	N	-	122.3	81.3	178.4
	U5[a]	4.18	N	-	75.2	75.2	136.7
	U6	2.56	N	-	31.7	31.7	62.8
Mean ± SD					76.4 ± 45.31	62.73 ± 27.05	126 ± 58.54
Only GnRH injected	U16[b]	4.89	-	N	212.2	50.7	284.4
	U17	4.23	-	N	26.97	5.1	26.97
	U18[c]	13.2	-	N	54.2	14.9	54.2
Mean ± SD					97.79 ± 100	23.57 ± 24	121.9 ± 141.4

*The blood samples from this lion was only analysed with the Siemens RIA. The reported testosterone concentrations for this lion were calculated as 33.5% of the concentrations measured by the Siemens RIA (See Section 5.2.3.1 for motivation).

Control testosterone profile

The testosterone concentrations of the control male (Figure 5.8) initially increased up to 40 minutes into the sampling window. After this, testosterone decreased relatively constantly up to 130 min of sampling and stayed constant at the basal concentration for the rest of the sampling time. This lion received only one top-up sedation dose between 140 and 150 minutes into the sampling procedure (2 hour and 50 minutes after initial sedation).
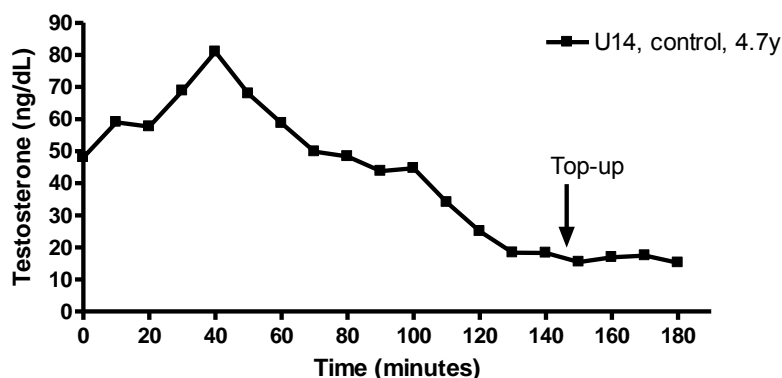
Figure 5.8: Testosterone profile over time for the control male (IBL RIA). Classified as young-adult (Brown) or adult (KNP). Time of anaesthetic top-up indicated. Initial anaesthetic administered at -30 min.

Testosterone responses to Kp-10 and GnRH stimulation: individual profiles

The testosterone response profiles of all the lions that received both Kp-10 (at time = 60 minutes) and GnRH (at time = 150 minutes) can be seen in Figures 5.9 to 5.14. Figure 5.9 shows the results obtained for lions NZG 04 (Figure 5.9 (A)) and NZG 07(Figure 5.9 (B)) as analysed with both the Siemens and IBL RIA's. While not exactly the same, similar testosterone response profiles for each animal were generated with the two RIA's. Using these data it was established that the IBL RIA gives a testosterone concentration result that is on average 33.5% of the testosterone concentration obtained by means of the Siemens RIA. The reasons for this may be due to constituents in the unextracted plasma samples interfering to varying degrees with the Siemens and IBL RIA's. See section 5.3.1.1 for results generated using this calculation.

One lion (NZG 10) subjected to the standard protocol did not show a testosterone response to Kp-10 or GnRH stimulation (Figure 5.10). A rapid increase in testosterone concentrations was observed between 20 and 30 minutes of sampling. This coincided with the time that this lion started to rapidly come out of anaesthesia before it could be intubated on Isoflurane. After receiving a top-up dose of anaesthesia the testosterone concentrations dropped again but not enough to properly observe the resultant testosterone response to Kp-10 stimulation. Note: A response to Kp-10 was considered if the testosterone concentration showed at least a 30% increase over the concentration measured just before Kp-10 administration at 50 minutes after Kp-10 administration. Whilst inspecting the results it was suspected that some adsorption of the GnRH could have occurred since the peptide transport vials, syringes, and catheter tubing used in the

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experiment were not coated with a protein such as for example BSA. Therefore, a broader classification for a GnRH response was used with a response to GnRH considered if the testosterone concentrations showed a sustained increase of above 30% for at least 30 minutes during the sampling window following GnRH administration.

Both lions U9 (Figure 5.11) and U10 (Figure 5.12) showed testosterone responses to Kp-10 stimulation but the response to GnRH was small (U9) or even lacking (U10). In order to stand a better chance of observing a testosterone response to GnRH stimulation, two lions (U20, Figure 5.13; U21, Figure 5.14) were subjected to an extended protocol (210 minutes of sampling instead of 180 minutes). In both cases the extended sampling time allowed for the testosterone response to GnRH to be observed. However, these males had different testosterone response curve profiles (Figures 5.13 and 5.14). For lion U20 the increase of testosterone in response to Kp-10 was followed by a drastic decline to a point lower than the lowest initial basal concentrations. After GnRH stimulation the testosterone concentrations then increased to the highest levels of this lions' response profile. On the other hand, the testosterone profile of lion U21 showed a continued gradual increase after Kp-10 administration, through GnRH administration, and to a point of drastic decline 40 to 50 minutes after GnRH stimulation. Additionally, lion U21 had the lowest reported baseline testosterone concentrations in this study while the magnitude of his response to Kp-10 and GnRH response was the greatest (~850% increase) of all the lions studied.
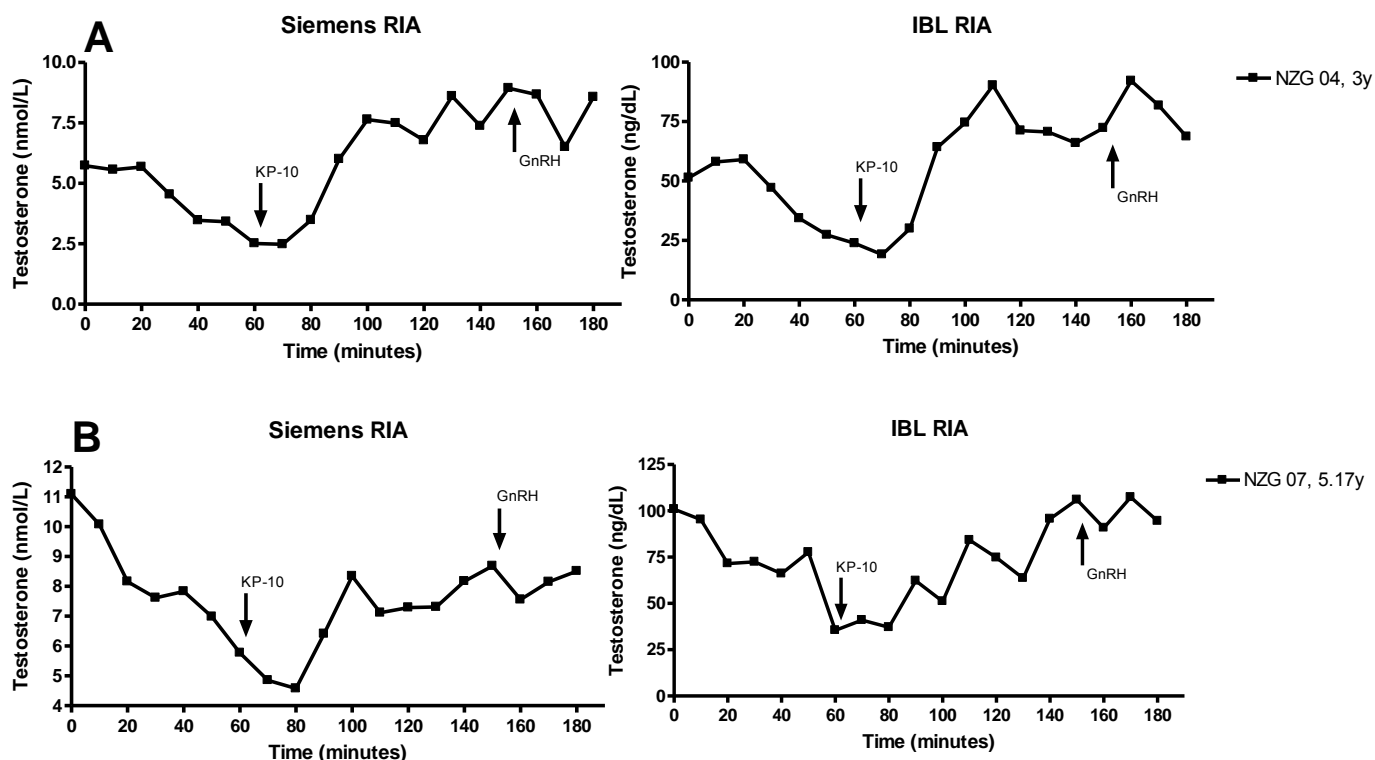
Figure 5.9: Testosterone response profiles over time for lions NZG 04 (A) and NZG 07 (B) as analysed with the Siemens RIA and the IBL RIA. NZG 04 classified as young-adult (Brown) or sub-adult (KNP). NZG 07 classified as adult (Brown and KNP). Initial anaesthetic administered at -30 min (NZG04) and -20 min (NZG07).

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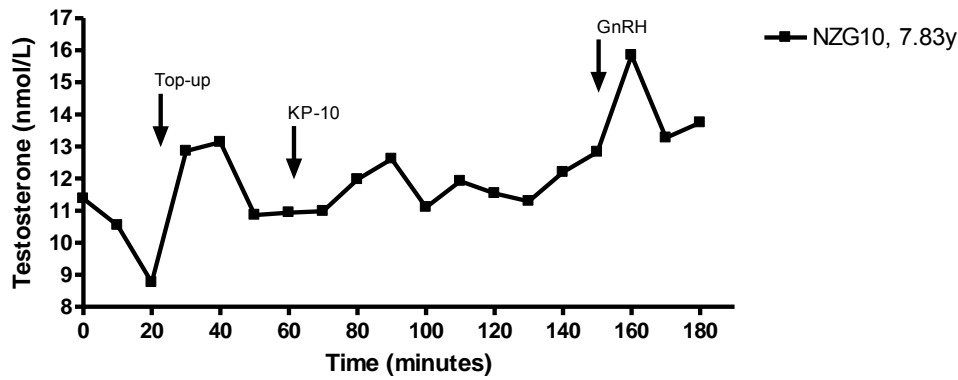
Figure 5.10: Testosterone response profile over time for lion NZG 10. The samples from this lion was only analysed with the Siemens RIA. Classified as adult (Brown and KNP). Initial anaesthetic administered at -25 min.
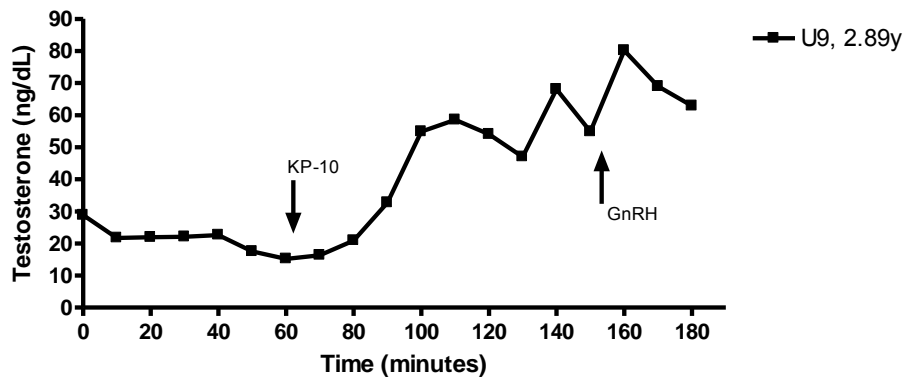
Figure 5.11: Testosterone response profile over time for lion U9. The samples from this lion was only analysed with the IBL RIA. Classified as young-adult (Brown) or sub-adult (KNP). Initial anaesthetic administered at -30 min.
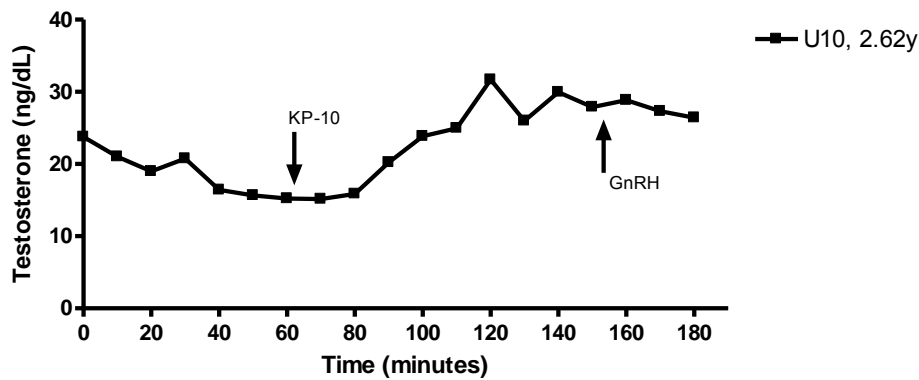
Figure 5.12: Testosterone response profile over time for lion U10. The samples from this lion was only analysed with the IBL RIA. Classified as young-adult (Brown) or sub-adult (KNP). Initial anaesthetic administered at -30 min.
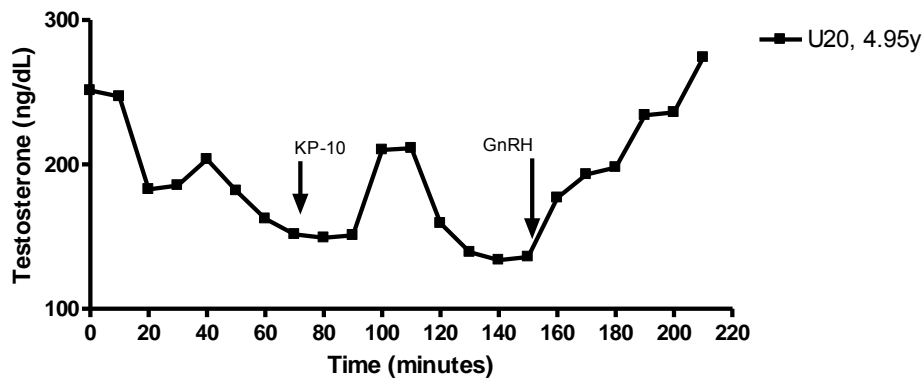
Figure 5.13: Testosterone response profile over time for lion U20. The samples from this lion was only analysed with the IBL RIA. Classified as young-adult (Brown) or adult (KNP). Initial anaesthetic administered at -50 min.
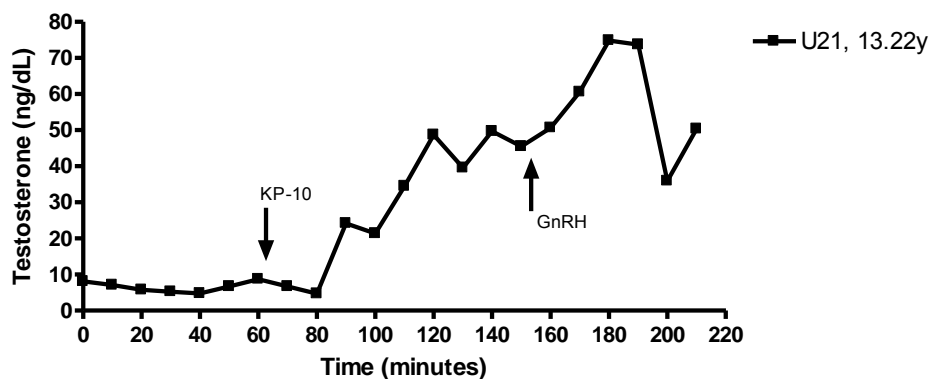
Figure 5.14: Testosterone response profile over time for lion U21. The samples from this lion was only analysed with the IBL RIA. Classified as adult (Brown and KNP). Initial anaesthetic administered at -50 min.

Testosterone responses to Kp-10 or GnRH stimulation

None of the lions that received only a Kp-10 dose (Figure 5.15) or a GnRH dose (Figure 5.16) showed a testosterone response. Figure 5.15 shows that the younger male (U6) had lower testosterone concentrations than the other two lions throughout the sampling procedure. For the GnRH only challenge (Figure 5.16) the highest testosterone concentrations were observed in the older of the two younger lions while the very old male had testosterone concentrations slightly higher than the youngest male. The 13 year old male (U18) had similar testosterone concentrations to that of the youngest lion (U17). Lion U16 (a.k.a. U20) had the highest basal and overall testosterone concentrations of all males used in this study. Lions U16 and U17 could be considered as young-adults (Brown) or adults (KNP).
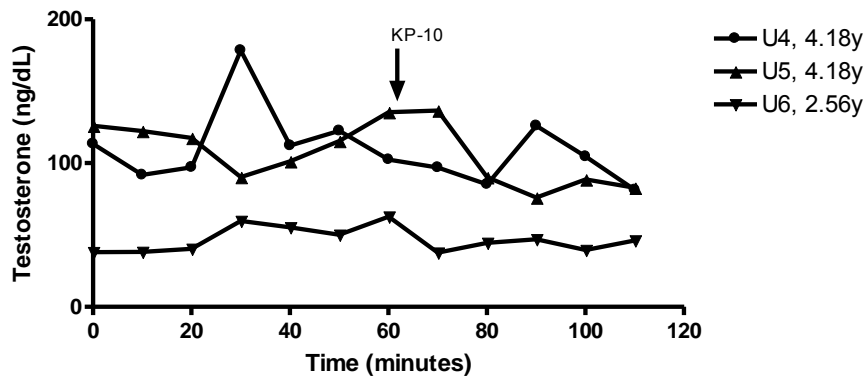
Figure 5.15: Testosterone response profiles for the three lions that received only a Kp-10 stimulation in the shortened sampling protocol. Lions U4 and U5 are classified as young-adults (Brown) or adults (KNP). U6 is classified as a young-adult (Brown) of a sub-adult (KNP). Initial anaesthetic administered at -30 min (U4, U5, and U6).
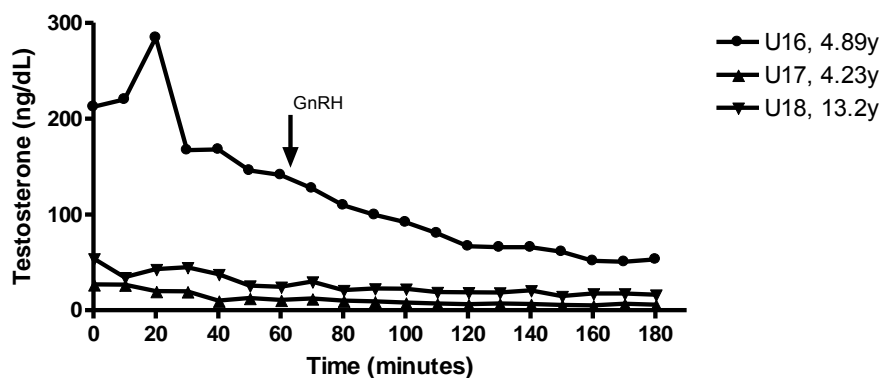
Figure 5.16: Testosterone response profiles as measured with the IBL RIA of the three lions that were subjected to only a GnRH stimulation. Lions U16 and U17 are classified as young-adults (Brown) or adults (KNP). Lion U18 is classified as an adult (Brown and KNP). Initial anaesthetic administered at -60 min (U16 and U17) and -50 min (U18).

Testosterone responses to Kp-10 stimulation in relation to age

Testosterone responses to Kp-10 were inspected taking the age classes into consideration and focusing on the sampling time window excluding the GnRH stimulation part of the experiment. Unfortunately, in many of the age classes there were not enough individuals to do comparative statistics. It should be noted that the age classification that incorporated the most individuals was the young-adult group (Brown system) with a total of four individuals. All other age groupings for which statistical data are reported only consisted of three individuals, bringing the meaningfulness of the statistical results into question.

According to the Brown age classification system young-adult lions show a greater mean response to Kp-10 stimulation than do adult lions (Figure 5.17, top). For the Brown classification

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system, mean testosterone concentrations 50 minutes after Kp-10 administration of young-adult lions were significantly greater (paired t-test, p = 0.0381) than before Kp-10 administration. Mean testosterone concentrations of non-responder young-adults did not differ significantly before and after Kp-10 administration (paired t-test, p = 0.1170). The opposite is seen with the KNP classification system where adult lions show a greater mean response to Kp-10 stimulation than sub-adult lions (Figure 5.17, bottom). Lions classified as adults according to the KNP system had significantly greater (paired t-test, p = 0.0330) testosterone concentrations 50 minutes after Kp-10 administration compared to before. The greater testosterone concentrations of sub-adult lions after Kp-10 administration were not significantly different from the concentrations before (paired t-test, p = 0.1296). Irrespective of the age classification system used, the Kp-10 non-responder lions showed a decrease in testosterone concentrations. Note: Kp-10 non-responders are lions that did not show a response to Kp-10 administration while properly sedated. In this regard lion NZG10 was not included in this classification since his lack of response could be ascribed to his sedation wearing off during the baseline sampling period preceding Kp-10 administration.

The time windows used for AUC calculations were 50 minutes before and after Kp-10 administration for the non-responder lions while for the rest of the lions 60 minute time windows were used. The AUC of each age group was calculated using the combined mean response curve of all the lions represented in each age group. Inspection of the AUCs showed that with the Brown age classification system (Figure 5.18, top) young-adults (responders and non-responders) had greater mean AUCs than adult lions. The overall AUC of non-responder young-adults (50 minutes before and after Kp-10 administration) did not differ significantly (unpaired t-test, p = 0.9626) form the overall AUC of the responder young-adults (60 minutes before and after Kp-10 administration). The AUC of young adults that responded to Kp-10 stimulation was similar before and after Kp-10 administrations while a slight decrease could be seen for adult and non-responder young-adult lions.

When lions were grouped according to the KNP age classification system (Figure 5.18, bottom) the overall AUC of adult lions (60 minutes before and after Kp-10 administration) were greater, although not significantly (unpaired t-test, p = 0.3964), than the overall AUC of sub-adult lions. An increase in AUC after Kp-10 administration was only seen in sub-adult lions while adult, and non-responder adults and sub-adults showed a decreased AUC.

The testosterone response profiles to Kp-10 stimulation were plotted according to age classes (Figure 5.19). With the Brown classification system young-adult lions had higher baseline testosterone concentrations and the peak testosterone response was greater than that of adults. The reverse was seen using the KNP age classification system. Additionally, the difference between baseline testosterone concentrations of adult and sub adult lions (KNP system) were greater than the difference between adult and young-adult lions (Brown system). One lion (U21) died due to septicaemia two months after sampling and, at the time of sampling, was experiencing much aggression from the females he was housed with. The testosterone response profile of this

male was plotted separately from the other adults in Figure 5.20. For the Brown age classification (Figure 5.20, left) this resulted in similar testosterone concentrations and response profiles for young-adult and adult males. The highest stimulated testosterone concentration of U21 was slightly higher than the lower baseline concentrations of the other adult male. Using the KNP age classification system (Figure 5.20, right) lion U21's testosterone concentrations were lower than that of the mean concentrations for sub-adult lions throughout the sampling procedure. Excluding this male from the adults also resulted in a greater difference in testosterone concentrations between sub-adult and adult lions throughout the sampling period.
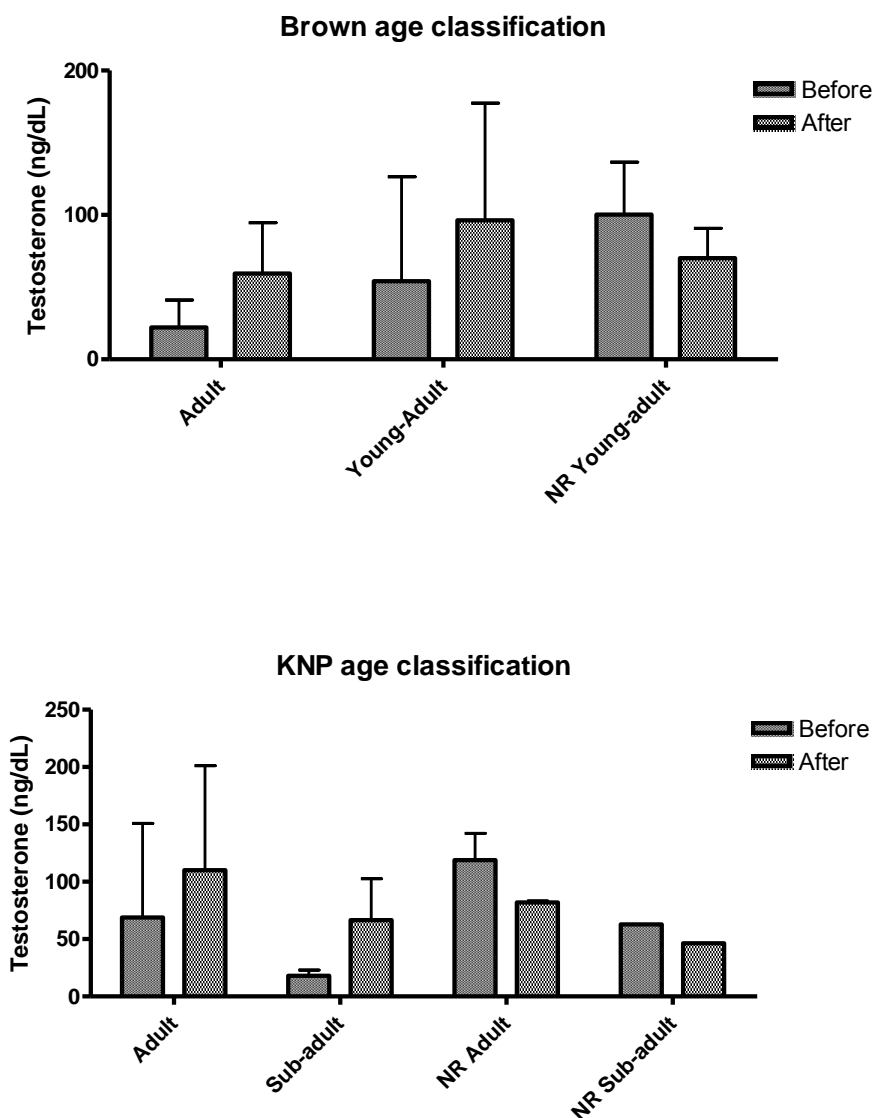
Figure 5.17: Histograms portraying the mean testosterone response to Kp-10 stimulation of the different age groups for the two age classification systems. (Before =Testosterone concentration just before administration of Kp-10; After = Testosterone concentration 50 minutes after Kp-10 administration; NR = non-responders; Vertical lines indicate standard deviation).

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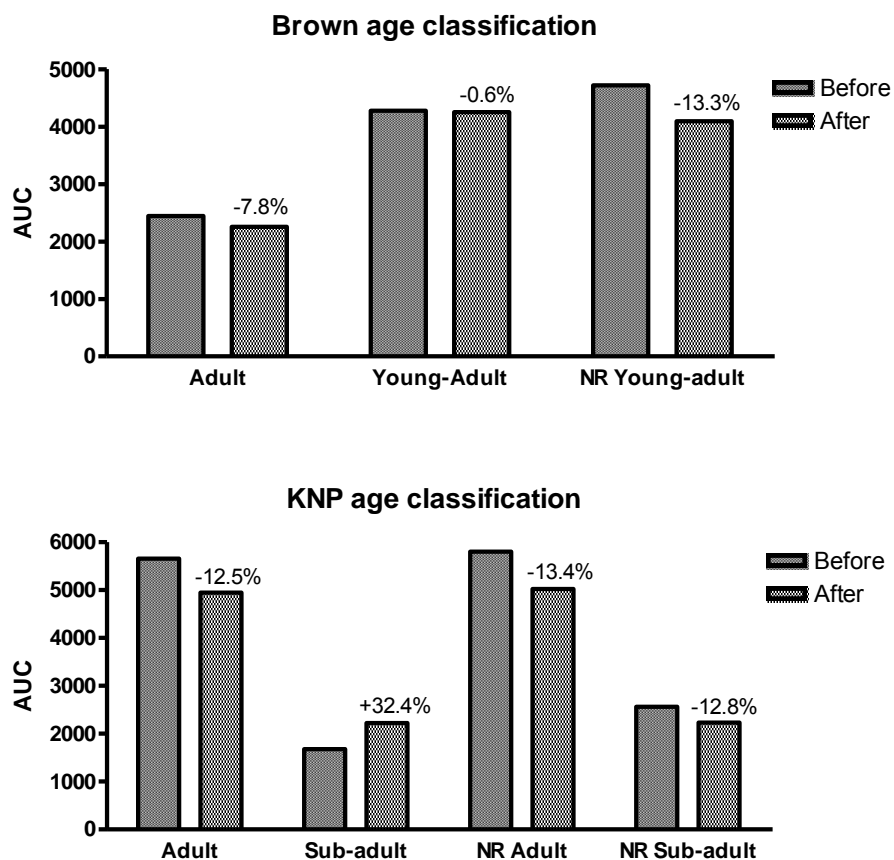
Figure 5.18: Histograms of the AUC comparisons for the different age classes according to the two age classification systems. (Before = mean AUC of testosterone profiles for the 60 minutes prior to Kp-10 administration; After = mean AUC of testosterone response profiles for the 60 minutes following Kp-10 administration; NR = non-responders, mean AUC for NR lions calculated with the testosterone response profile for 50 minutes prior and 50 minutes following Kp-10 administration). Proportional differences between AUC before and after Kp-10 administration are indicated.
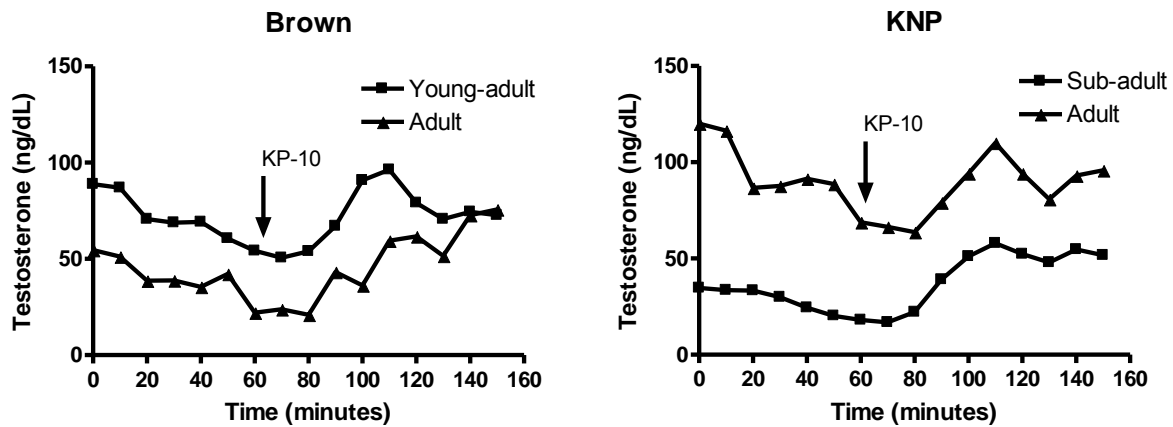
Figure 5.19: Mean testosterone responses profiles to Kp-10 stimulation according to age classes. Standard error of mean bars not shown for clarity purposes. (Brown young-adult = NZG04 + U9 + U10 + U20, Brown Adult = NZG07 + U21, KNP Sub-adult = NZG04 + U9 + U10, KNP Adult = NZG07 + U20 +U21)
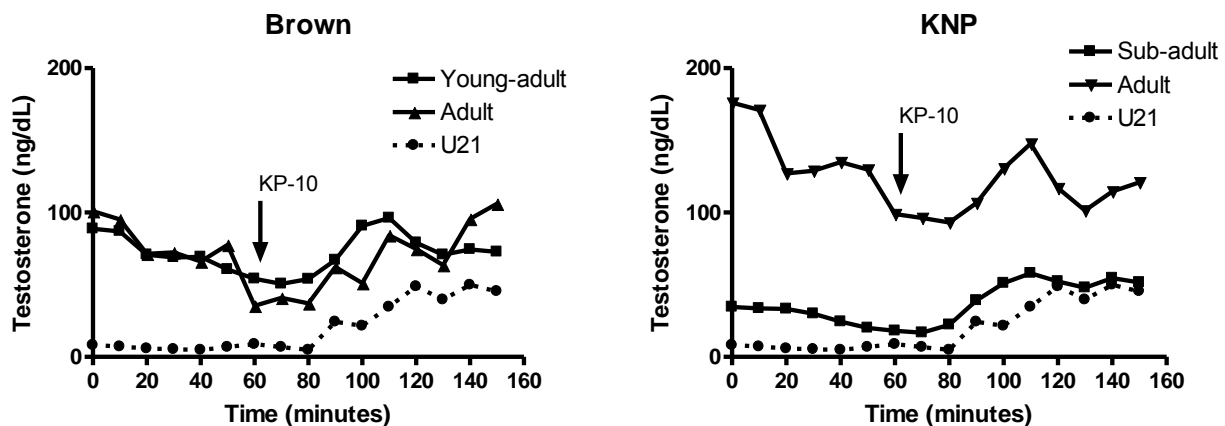
Figure 5.20: Mean testosterone responses profiles to Kp-10 stimulation according to age classes. Lion U21 is plotted separately from the other adults. Standard error of mean bars not shown for clarity purposes. (Brown young-adult = NZG04 + U9 + U10 + U20, Brown Adult = NZG07, KNP Sub-adult = NZG04 + U9 + U10, KNP Adult = NZG07 + U20)

Testosterone responses to GnRH stimulation in relation to age

The testosterone responses to GnRH stimulation were inspected by looking at the testosterone response profiles (Figures 5.16 and 5.23) as well as at the testosterone concentration just before GnRH was administered and at the concentration at 50 minutes after GnRH administration (Figure 5.21). This was done for the lions that received only a GnRH dose with no preceding Kp-10 administration, and for the lions that had a Kp-10 administration 90 minutes beforehand with sampling continuing until 60 minutes after GnRH administration. Because the lions subjected to the standard protocol had only samples taken for an additional 30 minutes after administration, the testosterone concentration of the last sample was used for comparisons. Additionally, the AUCs before and after GnRH administration were also inspected (Figure 5.22). For the lions subjected to only GnRH administrations and the lions subjected to the extended protocol a time window of 60 minutes before and after GnRH administration was used to calculate

the AUC. In the case of lions subjected to the standard protocol only 30 minutes before and after GnRH administration could be used.

According to the Brown age classification system (Figure 5.21) only the young-adult male subjected to the extended protocol showed an increase in testosterone after GnRH administration. When one considers the KNP age classification system (Figure 5.21) only the adult lions subjected to the extended protocol showed an increase in testosterone after GnRH administration. Irrespective of the age classification system used, none of the other males showed a testosterone response to GnRH stimulation.

The testosterone response profiles (Figures 5.13, 5.14, 5.16 and Figure 5.23) show that the only times testosterone responses to GnRH stimulation could be observed were in the males subjected to the extended protocol with a more pronounced response in the young-adult male (Brown classification). Both these males were classified as adult with the KNP age classification system. When looking at the AUC results (Figure 5.22), irrespective of the age classification system used, the lions subjected to only GnRH stimulation showed decreased AUCs after GnRH administration. All other lions, that had a preceding Kp-10 administration, showed increased AUCs after GnRH administration. Irrespective of the age classification system used, within the different treatment groups adult lions had a greater proportional AUC increase (decrease for the lions that received only GnRH) after GnRH administration than their younger counterparts. Additionally a proportionately greater increase in AUC was seen for the lions subjected to the extended protocol compared to the lions subjected to the standard protocol.

Brown age classification

KNP age classification
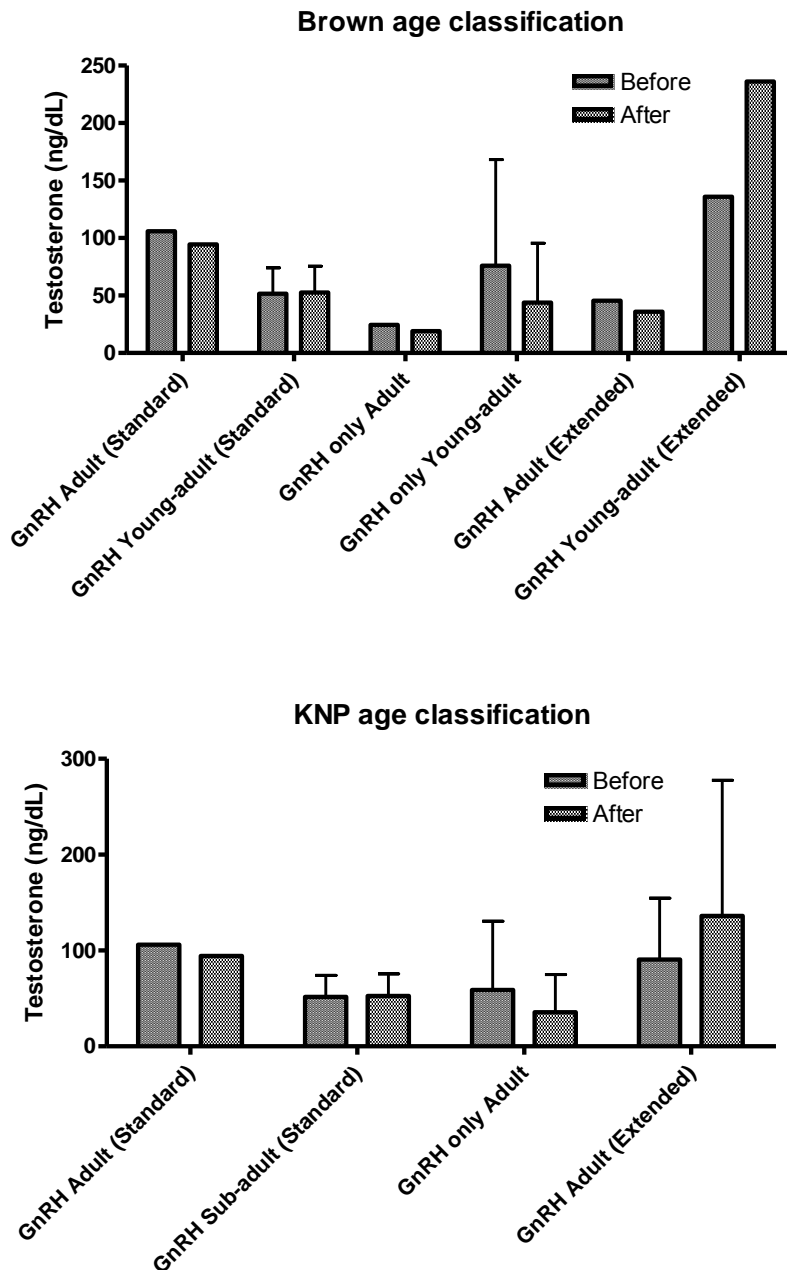
Figure 5.21: Histograms portraying the mean testosterone response to GnRH stimulation of the different age groups for the two age classification systems. (Before =Testosterone concentration just before administration of GnRH; After = Testosterone concentration after GnRH administration at 50 minutes for lion subjected to the GnRH only and the extended protocol, and at 30 minutes for lions subjected to the standard protocol; Vertical lines indicate standard deviation).
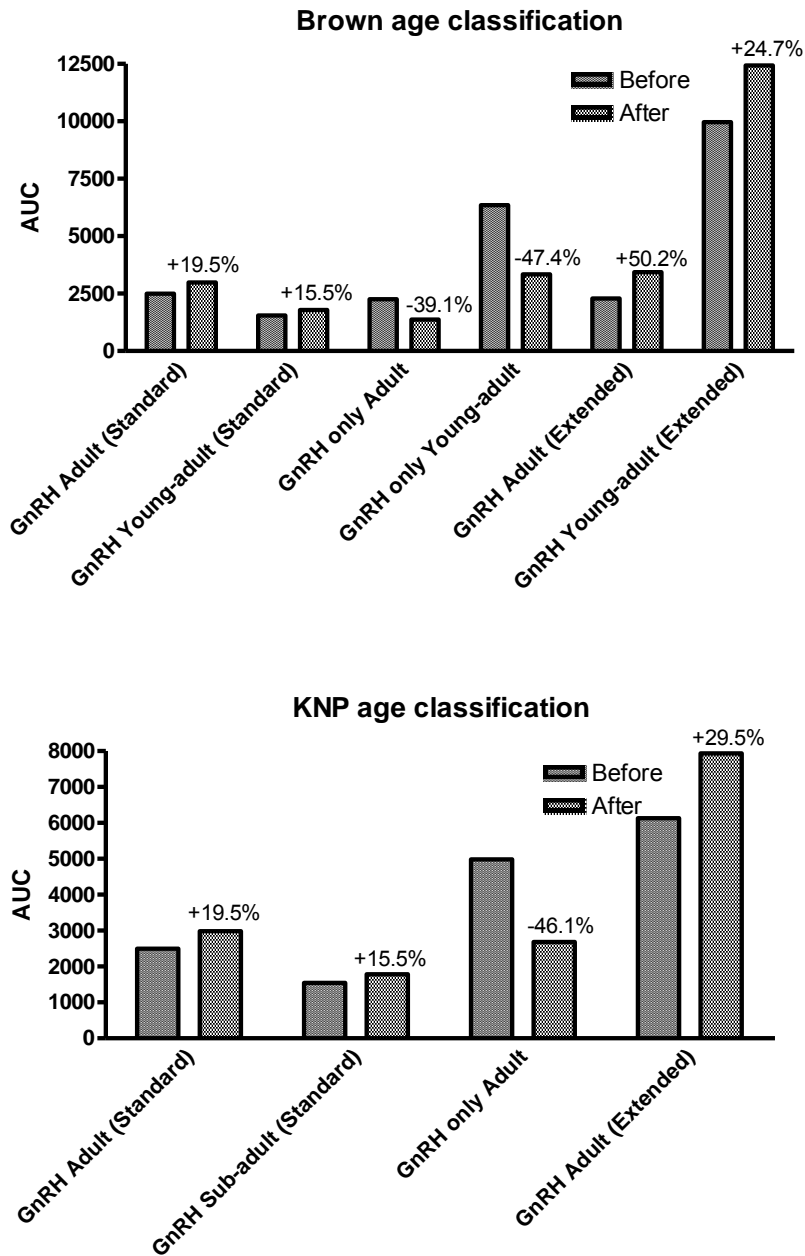
Figure 5.22: Histograms representing the AUC's calculated for the testosterone responses to GnRH stimulation for the different age classes according to the two age classification systems. (Before =AUC of testosterone profile prior to GnRH administration; After = AUC of testosterone response profile after GnRH administration. Time window for AUC calculation was 30 minutes before and after GnRH administration for lions subjected to the standard protocol, and 60 minutes for lions that received only GnRH as well as for lions subjected to the extended protocol). Proportional differences between AUC before and after GnRH administration are indicated.

Brown

KNP
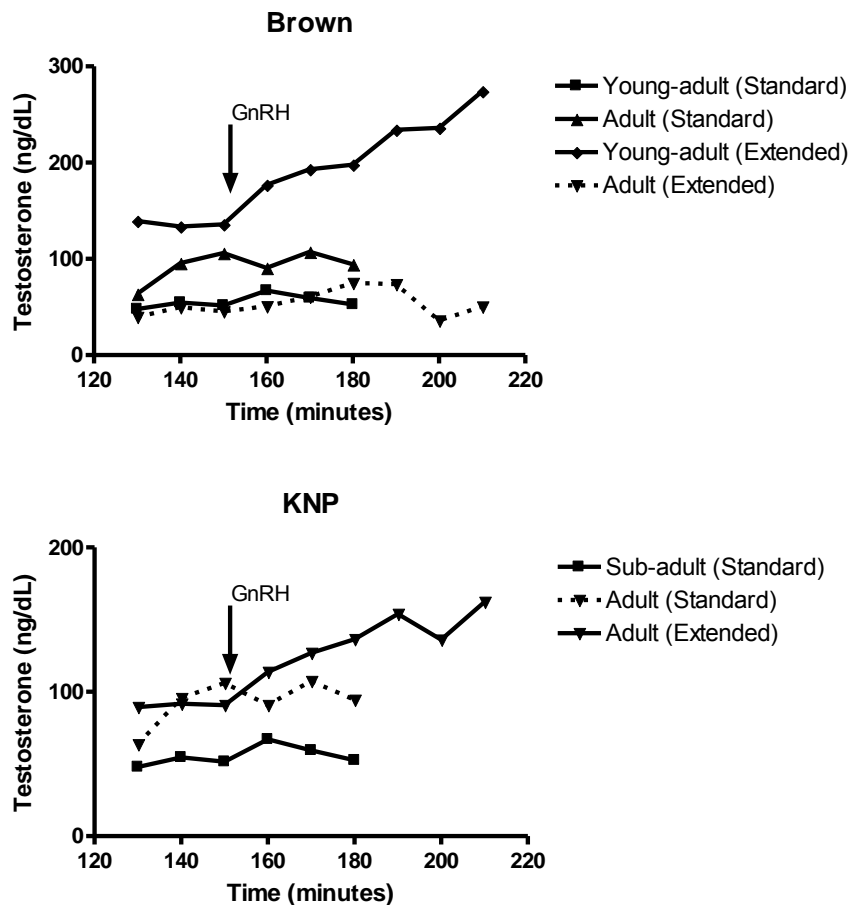
Figure 5.23: Mean testosterone responses profiles to GnRH stimulation according to age classes as per the Brown (top) and KNP (bottom) classification systems. All these animals had a Kp-10 stimulation 90 minutes before GnRH administration. Standard error of mean bars not shown for clarity purposes. Standard = standard sampling protocol; Extended = extended sampling protocol.

5.3.2.2 Progesterone

The progesterone response curves for four of the seven captive females subjected to the kisspeptin challenge experiment can be seen in Figure 5.24. The other three females had progesterone concentrations above the maximal level of quantification throughout the experimental procedure. These three lions consisted of one adult female (age = 5.17 years) and two sub-adults (age = 3 years). In general it does not seem as though Kp-10 or GnRH stimulates a progesterone response within the experimental time window. A slight decrease in progesterone concentrations over time was observed but was otherwise relatively stable. None of the fluctuations or average decreases in progesterone concentrations of the four individual lions exceeded two units of measurement (± 2 nmol/L) from the initial baseline sample. The lowest progesterone concentrations were measured in a 5.17 year old adult female.
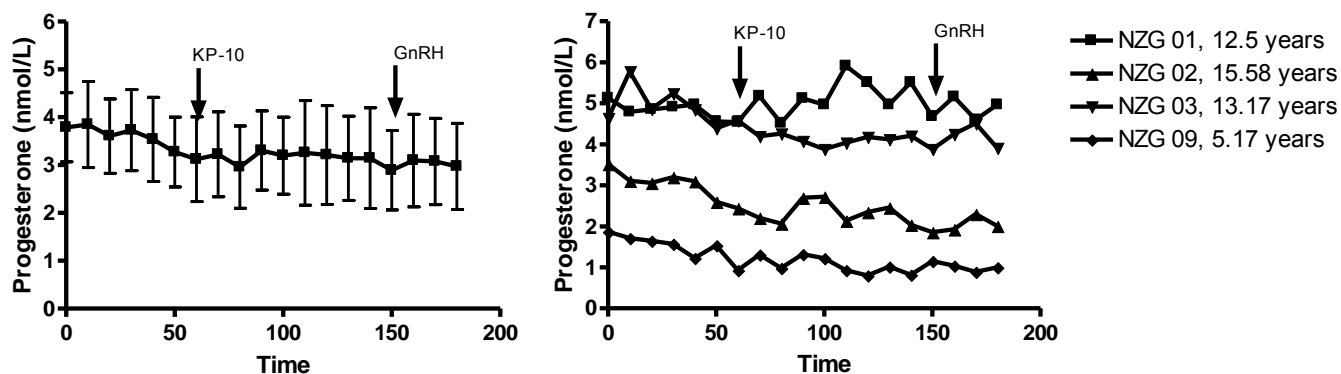
Figure 5.24: Progesterone response curves for four adult lionesses combined (left) and separate (right). Vertical lines indicate SEM. Age in years of the individual females are indicated.

5.4 Discussion

This study was aimed at firstly comparing reproductive hormone profiles of lions with suspected *M. bovis* infections with that of lions considered to be healthy or uninfected. Additionally, a novel method of interrogating lion reproductive endocrinology through the stimulation of the HPG axis by Kp-10 was also explored. For these purposes the main reproductive steroids namely oestradiol, progesterone, and testosterone were measured. Results for these hormones will be discussed in light of the *M. bovis* infection or disease status as well as for the usefulness of these hormones to interrogate lion reproductive endocrine function.

5.4.1 Testosterone

The majority of past research on the functioning of the reproductive system of lions has been focussed on females. Therefore, not much data are available for male reproductive hormones with which to compare the results of the current study with.

One of the major drawbacks in the current study was the discontinuation of the testosterone RIA (Siemens RIA) used to analyse the wild/KNP and some of the captive lion blood samples. Unfortunately, due to a limited volume of blood sample, all of the wild lion samples could not be re-run with the replacement RIA kit (IBL RIA), thereby making comparisons between absolute levels in wild and captive lions difficult. For both the Siemens and IBL RIA's comparisons of the sample dilution curve with the standard curve (see section 5.2.3.1) of the RIA showed that the testosterone concentrations obtained were likely representative of the circulating testosterone concentrations. When blood samples from two of the captive lions used in the kisspeptin experiment were run with both the Siemens and IBL RIA's it was established that the testosterone concentrations obtained by means of the Siemens RIA were on average 33.5% of the concentrations measured with the IBL RIA. Using this, it was possible to calculate an approximate IBL RIA testosterone concentration for the wild lions. Of the two RIA's the testosterone concentrations in the wild lions obtained with the Siemens RIA were comparable with that described in Brown et al. (1991). The calculated IBL RIA testosterone concentrations of the wild lions were much lower than the testosterone concentrations described by Brown et al. (1991). If one were to adjust the IBL RIA testosterone

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concentrations of the captive lions to that of the Siemens RIA, testosterone concentrations of captive lions would be higher than previously described for wild lions by Brown et al. (1991). Since the current study and that of Brown et al. (1991) made use of unextracted blood samples it is likely that much of this variation in testosterone concentrations between the different RIA's used may be due to constituents in the blood interfering to varying degrees within the different RIA's. Therefore, caution is paramount when comparing results from this study with that of previous studies.

The study by Brown et al. (1991) showed age related differences in testosterone and LH production in male lions. Therefore, age needed to be accounted for in the current study. However, the age classification system used by the KNP veterinary team differs from the age classification system used by Brown et al. (1991). This is due to Brown et al. (1991) classifying according to pituitary-gonadal development (as deduced from their study) while the KNP makes use of age. Both of these age classification systems were considered in the interpretation of the testosterone results. Since the Brown classification system is based on reproductive maturation it was decided to make use of both the KNP and Brown systems to interpret the testosterone data as it would likely help to better describe possible differences between the study populations as well as serve as a basis from which to compare current results with that of Brown et al. (1991). Differences in result interpretation occurred because of this and will be discussed later.

5.4.1.1 Single point testosterone concentrations

Unfortunately, subdividing the males into age classes resulted in some age classes being poorly represented for either the wild/ *M. bovis* exposed or captive/ *M. bovis* unexposed lions. This prevented meaningful statistical comparisons of lions in the same age class between populations as well as between age classes within the captive or wild populations. Despite not being able to establish statistical significance some general observations could be made.

Comparing age classes within lion populations

In the wild lion population, using the Brown age classification system, adults had higher total testosterone concentrations than young-adults which in turn had higher concentrations than pre-pubertal males (Figure 5.4). This is similar to the findings of Brown et al. (1991). Note: The KNP age classification system did not change the allocation of wild lions to the different age groupings (Table 5.1) and therefore testosterone concentrations were as follows, adults > sub-adults > juveniles. For captive lions the age classification system used (KNP or Brown) changed the allocation of individual lions to the different age groupings (Table 5.1). Using the Brown classification system young-adults had higher testosterone concentrations than adults while with the KNP classification system adult males had higher testosterone concentrations than sub-adult males (Figure 5.5).These differences in testosterone results, depending on the age classification system used, might be an indication that adult males in captivity have comparatively lower testosterone concentrations than their wild counter parts when compared to young-adult males. Alternatively it could be an indication that sexual maturation in captive lions occur earlier in life with

males producing testosterone concentrations at adult levels at a younger age. Much of this remains speculative at this time with the data available from the current study.

Comparing age classes between lion populations

Irrespective of the age classification system used, within the age classes, captive lions had higher testosterone concentrations than wild lions. Some possible reasons could be presented for this. The first might be that the lower testosterone concentrations in the wild lions are an artefact resulting from calculating a theoretical IBL RIA testosterone concentration from the Siemens RIA concentration. A second possibility might be that *M. bovis* infection in wild lions is having a negative effect on male lions' ability to produce testosterone. However, when looking at the individual lions (Table 5.2), this does not seem to be the case. Of the adult lions, the lowest testosterone concentration came from a likely uninfected individual while the highest concentration was seen in a likely infected male. The differences observed might therefore be due to processes other than *M. bovis* infection or disease. A possible explanation for this might be seen in the amount of time that passed between when the lion was non-responsive due to sedation and when the blood sample for testosterone analyses was taken. This becomes a factor when looking at the testosterone profiles reported in Brown et al. (1991) and in the current study (see following section). In general a decline in testosterone concentrations was observed over time from the point of the first sample to the time that the injected peptide hormone (Kp-10 or GnRH) illicit a testosterone response. This decline is likely due to inhibitory effects of anaesthetic drugs on endogenous HPG axis factors upstream of testosterone (Ching, 1982; Emanuele et al. 1987; Goubillon et al. 1999; Illius et al. 1983; Sherwood et al. 1980). The degree to which parts of the HPG axis can be inhibited and sometimes even stimulated varies between the types of anaesthetics used, the time passed since drug administration until sampling, the species studied, genders, and the phases/seasons of the reproductive cycle (Bizzaro et al. 1982; Brown et al. 1991; Ching, 1982; Goodman & Meyer, 1984; Matzen et al. 1987; Sherwood et al. 1980; Thompson et al. 1988; van Jaarsveld & Skinner, 1991). Therefore, the anaesthetic drugs used and the time delay until taking the first blood sample likely played a role in the testosterone results obtained in the current study. For example, in the current study, samples from wild lions in general were taken longer after the time of sedation than were samples from captive lions. Considering the decline in testosterone concentrations of ~70% in the first 70 minutes of sampling for captive lions (see section 5.4.1.2) and ~80% decline over time for wild lions described in Brown et al. (1991), it is possible that the extended time delay until sampling of the KNP lions occurred could have resulted in the lower testosterone concentrations observed.

Notes on age related sample size differences

The small number of wild adult and young-/sub-adult lions (Table 5.1) can be ascribed to the life history and social dynamics of wild lions. Typically, in the wild a single male lion or a coalition of male lions will be dominant over a home range that can include one or more female

prides. The time a male is dominant usually is enough to sire at least one or two generations of cubs. Any male cubs that reach sexual maturity (i.e. puberty at approximately two years of age) are not tolerated with the rest of the pride and tend to be nomadic. Therefore, during the baited call-up (See Chapter 2 section 2.2.2) of wild lions, sampling bias occurred towards lions present in the pride while nomadic males or males patrolling their territory away from the pride were not sampled. In the case of the captive lions, a selection bias occurred when only adult and young-/sub-adult males were selected for the Kp-10 HPG-axis challenge experiment. Since, in the wild, lions of these age classes are more likely to be sexually mature and therefore the suspected negative effect of TB on reproduction would likely be present in them, these age classes and not juveniles were selected to serve as a base from which to do future reproductive endocrine investigations in wild lions under pressure from *M. bovis.*

5.4.1.2 Testosterone response to Kp-10 and GnRH challenge

Part of this study was focussed on developing a novel method to investigate the level of activity of the reproductive neuro-endocrine system of lions. It has been well documented and suggested that much of the inhibitory actions of external and internal inputs on the reproductive endocrine system of mammals are mediated through KP reduction (Gianetti & Seminara, 2008; Pinilla et al. 2012; Roseweir & Millar, 2009; Tena-Sempere, 2010). Reduced Kiss-1 production leads to reduced GnRH, LH, and ultimately reproductive steroid hormone production. We might therefore predict that in lions experiencing a negative energy balance or disease stress, inhibition of the KP-system might occur as described previously for rats (Castellano et al. 2005; Castellano et al. 2010) and mice (Luque et al. 2007). Thus, when the system is challenged with exogenous kisspeptins or GnRH, it will likely result in an exaggerated response compared to healthy lions in which the KP system has not been inhibited. Since endogenous GnRH (and KP) production appears to be inhibited during sedation of the lion, it provides a 'clean slate' on which to observe the response of the HPG axis to exogenous Kp-10 and/or GnRH stimulation without variable endogenous input. Furthermore, a variety of mammalian models showed that the KP system plays an integral role in sexual maturation and the onset of puberty (Clarkson et al. 2010; Pinilla et al. 2012; Roseweir & Millar, 2009). In this regard progression into puberty is associated with increased *Kiss1* expression and KP production, increasing concentrations of KP neuron fibres around GnRH neurons, increased electrical response duration of GnRH neurons after GPR54 activation, and/or increased sensitivity of the GPR54 receptors in GnRH neurons (Clarkson et al. 2010; Pinilla et al. 2012; Roseweir & Millar, 2009). One can therefore expect to see a lesser response to an acute exogenous Kp-10 stimulation in sexually immature lions than in adult lions. Interestingly, in this regard, results from the current study differ depending on the age classification system used (Figures 5.17, 5.18, and 5.19) with the KNP classification system showing the expected picture of sub-adult captive lions having lower basal testosterone concentrations and a lesser testosterone response to Kp-10 stimulation than did adults. Using the Brown age classification system the

inverse was seen. This further supports the suggestion mentioned in Section 5.4.1.1 that captive lions, in the current study, might be reaching sexual maturity earlier in life than do wild lion as described in Brown et al. (1991). Much of the knowledge on the post natal development of the KP system was derived from rodent studies (Clarkson et al. 2010; Pinilla et al. 2012) and the possibility of species differences in pubertal development of the KP/GPR54 system exists. Indeed, differences between species are well known for the overall neuroendocrine mechanisms in puberty onset (Pinilla et al. 2012)

The study by Brown et al. (1991) made use of GnRH to interrogate the HPG axis functioning. However, a stronger regulation of the HPG axis occurs at the sites of KP neurons than at GnRH neurons (Roseweir & Millar, 2009). Additionally, as mentioned before KP neurons are situated upstream of GnRH neurons and are responsive to many more inputs than GnRH neurons (e.g. reproductive and stress steroid hormones, inflammatory and growth factors). Therefore a provocative KP test might be more revealing than a GnRH test for investigation of HPG axis functioning.

In the study by Brown et al. (1991) a GnRH dose of 1 µg/kg body mass resulted in increased testosterone production between 15 to 50 minutes after intra venous (IV) injection. In the current study upon visual inspection of the testosterone response profiles (Figure 5.23) and the response histograms (Figure 5.21), despite making use of the same dose of GnRH, none of the lions subjected to the standard protocol (See Section 5.2.2 for description of protocols) showed an increase in testosterone after IV GnRH injection. This lack of a response to GnRH could possibly be ascribed to blood samples being taken only 30 minutes after the GnRH injection, thereby limiting the time to see the appropriate response. In order to address this, three lions were subjected to a GnRH challenge only. Two of these three were also subjected to the extended protocol. None of the lions injected with only GnRH had an increased testosterone response despite sampling continuing for 120 minutes after GnRH injection. The only two lions that showed a testosterone response to GnRH were the two males that had a Kp-10 injection 90 minutes prior to GnRH administration with sampling continuing for 60 minutes after GnRH administration. This might therefore serve to suggest that indeed the sampling window of 30 minutes after GnRH injection was too short to observe the testosterone response in the lions used for the standard protocol. However, the fact that Kp-10 stimulation, that is situated upstream of GnRH in the neural pathway, resulted in a testosterone response as early as 15 minutes after stimulation makes this an unlikely explanation. This is further emphasised by the AUC results (Figure 5.22) showing a greater proportional increase in AUC after GnRH administration for the lions subjected to the extended protocol compared to the lions subjected to the standard protocol. Therefore, considering only the AUC results, it looks as though GnRH is eliciting a testosterone response when preceded by a Kp-10 stimulation. Additionally, the increased sampling time window of the extended protocol allowed for better observation of this response. However, this does not explain why no testosterone response was observed in the lions that received only GnRH. One possible

explanation might be found in the type of drug used to sedate the lions during the experiment. The study by Brown et al. (1991) made use of the drug Telazol to anesthetise their lions while the three males that received only GnRH were anesthetised with a drug combination consisting of Medetomidine and Ketamine. The mode by which such inhibitory effects on GnRH responsiveness might take place is unclear and beyond the scope of this study. If the drugs used on the three GnRH-non-responders indeed resulted in any inhibition of the pituitary it still does not explain why a response (although relatively small) was observed in the two lions subjected to the extended protocol or why these lions had an response to Kp-10 stimulation as all these lions (GnRH non-responder and extended protocol lions) received the same drug combinations. All of this together with the fact that similar doses of GnRH elicited a response in Brown et al. (1991) might be an indication that the GnRH used in the current study was inactive or that some other factor not controlled for in this study might be at play. Inactivity of the GnRH is very unlikely since mass spectrometry analysis confirmed the structure and GnRH is known to be very stable. A more likely scenario is that the GnRH was extracted from the solution by adhering to the walls of the vials, syringes, and IV line used to transport and administer the GnRH solution, thereby decreasing the concentration of GnRH injected in to the lions to a threshold dose only capable of eliciting a testosterone response in the presence of a preceding maximal Kp-10 stimulation which may have masked GnRH adsorption sites. In retrospect the GnRH should have been made up with a protein such as BSA which is common practice when administering low doses of peptides.

The general decrease in baseline testosterone concentrations of 71% of the lions during the first 70 minutes of sampling in this study was similar to the ~80% described by Brown et al. (1991). This is also similar to a decline in testosterone concentrations of between 31% and 74% over 60 minutes in domestic cats anaesthetised with Ketamine (Carter et al. 1984). From the results of the current study a mean half life for total testosterone in male lions was calculated at ~50 minutes.

An increase in testosterone was observed between 40 and 60 minutes after IV injection of Kp-10 in six out of 10 lions. The other four lions did not show the decrease in testosterone during baseline sampling or an increased testosterone response to Kp-10 stimulation. For three of these four lions a possible explanation for the lack in testosterone responses can possibly be explained by the drug combination used since these three lions received a Medetomidine and Zoletil combination instead of a Medetomidine and Ketamine combination. This possibly resulted in a lesser inhibition of the neural pathways active in testosterone production. Since basal testosterone concentrations did not decrease during the initial 70 minutes of sampling, the increase of testosterone in response to Kp-10 stimulation could not be observed. This could be considered similar to the findings reported by van Jaarsveld & Skinner (1991) when a provocative test of the HPG axis of Spotted Hyenas (*Crocuta crocuta*) with GnRH also could not observe a testosterone response in animals anaesthetised with Zoletil and maintained with halothane due to testosterone levels staying relative consistent throughout their sample procedure. They suggested that for

provocative tests of the pituitary of Spotted Hyenas, other immobilising agents should be investigated and/or the time of anaesthesia should be prolonged in order to get a better resolution in the testosterone response profile (van Jaarsveld & Skinner, 1991). Alternatively, this could also be due to a possible inhibition of the GnRH neurons caused by Zoletil since pre-treatment with GnRH antagonist can abolish any LH, and therefore testosterone, stimulation by KP (Gianetti & Seminara, 2008; Pinilla et al. 2012; Roseweir & Millar, 2009; Smith et al. 2006). Candidates that could cause such inhibition are RFamide peptides (RFRP's: mammalian orthologs of avian gonadotropin-inhibitory hormone (GnIH)) (Pinilla et al. 2012) that could possibly be activated by Zoletil. However, this does not explain why there were no baseline declines of testosterone for these specific individuals during the initial 70 minutes of sampling.

The fourth lion that did not show a testosterone response to Kp-10 originated from the NZG. In the case of this lion, a relatively long time passed between the time of darting and the time that the lion could be intubated on Isoflurane. The result of this was that the lion started to wake up. Inspection of the testosterone profile (Figure 5.10) of this lion showed an initial decline in basal testosterone concentrations until the time that the anaesthesia wore off. This was followed by a sharp increase in testosterone to above the initial baseline level. Therefore, testosterone concentrations did not decrease enough by the time of Kp-10 administration to observe the resultant testosterone response. Additionally, this might also be an indication of some form of increased testosterone response due to handling stress since the increase in testosterone concentrations coincided with the time that the lion became responsive to stimuli. Further investigation will be needed.

The testosterone response curves to Kp-10 of the four lions subjected to the standard protocol were comparable to that described previously by Brown et al. (1991). The adult male had a higher initial baseline testosterone concentrations and a greater testosterone response to Kp-10 than the young-adult males. The testosterone concentrations (as measure by the IBL RIA) and response curves were similar to that measured by Brown et al. (1991) for both the adult male and the young-adult males. However, if one were to calculate a Siemens RIA testosterone result for the captive lions, the testosterone response curves, although similar in profile, would have greater testosterone concentrations than previously described by Brown et al. (1991) using a GnRH challenge.

Interestingly, the testosterone concentrations of the two males subjected to the extended protocol were not comparable to that described by Brown et al. (1991). Using the Brown age-classification system the young-adult male had testosterone concentrations much higher than previously described by Brown et al. (1991) while the older male (U21, Figures 5.14 and 5.20) had very low concentrations compared to adults in the current study and previously described. This old male was 13.2 years old at the time of sampling and died two months later of septicaemia. However, when this same male was sampled nine days earlier (receiving only GnRH) his initial baseline testosterone concentration was 6.7 time higher than his baseline concentrations at the

second sampling event. This, together with the fact that this male had the greatest proportional testosterone response to Kp-10 during the extended protocol could serve as an indication that basal testosterone production was reduced due to disease and/or environmental stress, resulting in the proportionally greater testosterone response. This is in line with the hypothesis that inhibition of the KP system due to disease would result in a greater response of the HPG axis to exogenous Kp-10 or GnRH stimulation when compared to healthy animals. During this time this male was still housed with a pride of females that he was with for more than four years prior. However, the caretaker of this pride reported that the females started to target and attack the male and it seemed as though they were trying to evict him from their territory during the later months. Whether this was due to the females being aware that he was declining in masculinity or had health issues that they perceived as a weakness, remains speculative.

When looking at only the AUC results (Figure 5.18) it would seem as though only sub-adult lions (KNP classification) showed a testosterone response to Kp-10 administration. However, considering the testosterone response profiles (Figures 5.19 and 5.20) and response histograms (Figure 5.17), showed that this was not the case. This decrease in AUC after Kp-10 administration can be ascribed to the fact that initially, after Kp-10 injection, testosterone levels continued to decrease until the stimulatory effect of Kp-10 could be translated by the gonads. This should not be confused with the slight decrease in testosterone (Figure 5.15) and therefore decreased AUC of the non-responder lions after Kp-10 administration. The increased AUC for the sub-adults (KNP classification) can be ascribed to a more pronounced testosterone response to Kp-10 stimulation, although still at lower concentrations than was seen in the adult (KNP classification) lions.

The study by Brown et al. (1991) was able to identify three main stages of reproductive development in male lions. As mentioned before, the testosterone response profiles of the current study was inspected considering both the Brown and the KNP age-classification systems. When the results of the unhealthy male (U21) was excluded a greater differentiation could be seen between adult and sub-adult lions according to the KNP classification than between adult and young-adult lions according to the Brown classification. This was largely due to one young-adult male (Brown classification) that could be classified as an adult by means of the KNP system. This lion was subjected to the GnRH only protocol as well as the extended protocol and in both instances had the highest testosterone concentrations of the lions in the respective groups as well as overall for all captive lions included in the neuro-endocrine study. At the time of the GnRH protocol this male was 4 years and 10 months old and one month older at the time of the extended protocol. It should be noted that the testosterone concentrations of his 60 minute baseline samples were on average slightly higher when he was one month older. Additionally, he was housed with a pride of females whereas the other young-adult (Brown classification) males that could also be classified as adults (KNP classification) were housed together and not with a female pride. Considering all of this together with the proposition that captive lions are reaching sexual maturity

earlier in life (See section 5.4.1.1), it might be speculated that other factors (such as social dynamics) in addition to age are contributing to male sexual maturation.

5.4.2 Progesterone

Progesterone concentrations previously reported for female lions were low throughout most of the oestrus cycle with peaks after ovulation or increased during pregnancy and a gradual decline as pregnancy progressed (Schmidt et al. 1979). Progesterone concentrations of non-pregnant females in the current study were lower than previously reported for non-pregnant female lions. This might be due to the use of a human progesterone RIA in the current study. Therefore, while progesterone concentrations reported in this study can give an idea of how the different lion populations compare, it might not be reporting the actual concentrations and can therefore not be compared to other studies. As mentioned in Section 5.2.3.3, progesterone concentrations above 31.8 nmol/L are likely not representative of circulating progesterone since the sample dilution curve was not parallel to the standard curve at concentrations > 31.8 nmol/L and constituents in the samples were likely interfering with the RIA assay. However, all of the lions that had progesterone concentrations higher than 31.8 nmol/L were also higher than the upper limit of detection for the assay (127.2 nmol/L). A total of six lionesses (three wild/KNP and three captive lions) had progesterone concentrations higher than the limit of detection of the assay. The wild/KNP lions with these high progesterone concentrations were all suspected to be pregnant after physical examination by the KNP veterinary staff that included observations of enlarged nipples and extended abdomens. This is in line with previous reports of progesterone concentrations in a pregnant female that ranged between 57.24 nmol/L and 457.92 nmol/L (Schmidt et al. 1979). One wild/KNP female suspected to be pregnant at the time of physical examination had a progesterone concentration of 5.9 nmol/L suggesting that she might not have been pregnant.

Previous studies reported progesterone concentrations between 159 and 318 nmol/L for pseudo-pregnant females and 54.06 to 896.76 nmol/L in females considered to have ovulated (Schmidt et al. 1979). In the current study none of the wild females that were not suspected of pregnancy had high progesterone concentrations. The only non-pregnant females with high progesterone concentrations were the three captive lions of which two were housed together and the third one in the adjacent camp (lions could see each other through an open mesh fence and could be within a two meter vicinity of each other). Since synchrony of ovulation has been reported in lions, it is possible that the high progesterone concentrations in the three captive females are indicative of a synchronised ovulation event preceding the time of sampling (Sampling occurred within a six day time window). It should be noted that these females were housed with vasectomised males and no behavioural observations were made in the time before or after sampling to confirm if ovulation was spontaneous or induced by means of copulation.

5.4.2.1 Progesterone response to Kp-10 and GnRH challenge

Similar to what was seen for GnRH stimulation by Brown et al. (1993), progesterone concentrations in the current study were not influenced by the Kp-10 and GnRH challenge (Figure 5.24) of the reproductive endocrine system.

5.4.3 Oestradiol

Results from a previous study showed that oestradiol production in female lions are cyclic in nature with intervals between cycle peaks varying within and between individual animals (Schmidt et al. 1979). The study by Brown et al. (1993) reported serum oestradiol concentrations that ranged from 7.9 to 51.4 pg/ml. Schmidt et al. (1979) reported low basal concentrations (< 14 pg/ml) with peaks ranging between 19 and 108 pg/ml. Both of these studies made use of radioimmunoassay (RIA) to measure oestradiol concentrations. In the case of Brown et al. (1993) they made use of a human oestradiol RIA. For the current study a human oestradiol RIA was also used, however, all of the results were below the limit of detection of the assay (< 20 pg/ml). Considering the lower limit of detection of the RIA used in the current study as well as the oestradiol concentrations reported in previous studies it seems possible that the oestradiol concentrations in the sampled females were all too low to be analysed with the RIA kit used. Alternatively, whilst this RIA has been established to measure oestradiol in human blood, interference by constituents of lion plasma might be hampering measurement of oestradiol in lion blood. It is therefore possible that had the samples gone through an extraction step with solvents such as ether, measurements might have been obtained. Comparisons could therefore not be made between animals of different *M. bovis* exposure/infection groups.

Due to the cyclic nature of oestradiol production as well as the fact that Brown et al. (1993) did not report effects of GnRH stimulation on oestradiol production within a two hour window after stimulation, it was decided not to pursue further blood oestradiol analyses.

5.5 Conclusion

The majority of previous studies on lion reproductive systems were focussed on the female reproductive system with the aim of describing the lion oestrus cycle. To my knowledge no studies have attempted to compare the reproductive abilities of lions in light of an external stressor such as disease. There is, however, one study (reported in two publications, one for each gender) (Brown et al. 1991; Brown et al. 1993) that compared the reproductive endocrine function of genetically inbred and out-bred lion populations in an attempt to explain the reduced breeding success of the inbred population.

To my knowledge, the current study was the first attempt to compare the reproductive abilities (at neuro-endocrine level) of a presumed disease stressed lion population and that of presumed healthy lions. This was also the first study to describe kisspeptin stimulation of the HPG axis of lions. Previous studies have reported effects of KP's on the HPG axis of rodents (rats and mice), primates (human and non-human), sheep, pigs, cows and dogs (Albers-Wolthers et al.

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2014; Gianetti & Seminara, 2008; Pinilla et al. 2012; Smith et al. 2006; Suzuki et al. 2008; Tena-Sempere, 2010).

Despite encountering difficulties with the hormone assays used and small sample sizes the following observations can be made by looking at results of the current study as well as from previous studies.

The analyses of the female reproductive steroid hormones oestradiol and progesterone are useful tools to assess the reproductive state of a female lion in terms of the oestrogen cycle as well as pregnancy. However, because production of these hormones are subject to the oestrus cycle and are unaffected by GnRH and/or Kp-10 stimulation within the sampling time window available, the use of these hormones to gain insights into the functioning of the reproductive neuro-endocrine system is limited. It was therefore not possible in the current study to ascertain whether female lion reproductive abilities are compromised by the presence of *M. bovis* in the wild lion population.

 In the case of male lions, analyses of testosterone concentrations can give insights into the stage of reproductive development of individual lions. Due to the problems encountered with the testosterone RIA it was not possible to make definitive conclusions about the reproductive abilities of the wild/*M. bovis* exposed population compared to the captive/unexposed population. It was, however, possible by means of an acute kisspeptin stimulation to distinguish between testosterone responses of captive adult and sub-adult lions as well as between captive healthy and ill/stressed (lion U21) adult lions. This has never been done before with KP in lions.

The current study showed the importance and need to standardise the sampling procedure (i.e. time between sedation and sampling) as well as the anaesthesia protocol (i.e. drugs used to sedate the lions as well as to keep them under anaesthesia), especially when working with different lion populations. This is also true for other animals as any sedation that works on the neurological system could possibly directly or indirectly affect the HPG axis. Additionally, the logistical challenge of working with wild animals, especially lions, need to be considered in the development of a standardised sampling protocol.

This study showed that Kp-10, similar to GnRH, has the ability to stimulate testosterone production in male lions. Considering the upstream position of KP neurons to GnRH neurons and the fact that much of the regulation of the reproductive cascade through exogenous and endogenous stimuli occur at the site of KP action, supports further investigation into the use of the KP system to interrogate reproductive endocrine functioning in organisms subjected to environmental stressors. The results obtained from lion U21 that showed an exaggerated response to Kp-10 stimulation in an unhealthy animal further supports this point.

As mentioned before, the reproductive steroid hormones are able to give some insights into the reproductive status of lions. In addition to the effect of the oestrus cycle on oestradiol and progesterone, the extreme downstream point in the reproductive cascade of the endocrine glands responsible for the production of these hormones further limit their usability in assessing the upstream reproductive neuro-endocrine state of an animal. Similarly the downstream location of

the testes also limits the use of testosterone for upstream assessments. Additionally, the variation in concentration of LH receptors in the testes throughout the sexual development of males (Brown et al. 1991) further limits the use of testosterone. On the other hand, production of LH occurs in the anterior pituitary lobe of the brain and should therefore give a better representation of the reproductive neuro-endocrine state of an animal, irrespective of the use of Kp-10 or GnRH. Furthermore, according to the findings by Brown et al. (1991) baseline circulating concentrations of LH are relatively stable and low over time. This can aid in developing a shorter sampling protocol to assess reproductive neuro-endocrinology in wild lions. This, together with the fact that the LH response to GnRH stimulation (and therefore likely Kp-10 also) is quicker than the testosterone response (Brown et al. 1991) and that an LH response was observed in females (Brown et al. 1993) further highlights the feasibility of using this hormone to asses reproductive neuro-endocrine function. Unfortunately, development of a LH ELISA able to recognise lion LH could not be completed before submission of this thesis. Further attempts to develop such an ELISA are underway.

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5.6 References

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Chapter 6

Multiple systems approach and conclusions

6.1 Introduction

The symptoms that accompanied the first cases of *M. bovis* infections in both captive and wild lions were emaciation, immobility and difficulty in breathing (Eulenberger et al. 1992; Keet et al. 1996; Morris et al. 1996). Necropsies performed on these index cases and subsequent cases revealed pathology in various organ systems (see Chapter 1 section 1.1). Additionally the chronic nature of the infection and disease most likely elicit different immune responses at different stages of the infection or disease, ranging from acute localised immune/inflammatory responses to chronic systemic responses.

Conditions of chronic immune activation and inflammation are often associated with a loss of appetite and a significant decline in body mass (Baatar et al. 2011). The reviews presented in many of the previous chapters alluded to the interlinked nature of the energy metabolism, immune/inflammatory response, and reproductive systems. This interconnectedness of the systems and the presence of clinical manifestations and pathology in a variety of organ and metabolic systems have lead to multifaceted investigations into disease progression and pathological processes of human TB. Some examples of such studies can be seen in Santucci et al. (2011), del Rey et al. (2007), and the review by Bottasso et al. (2010). Santucci et al. (2011) did a multifaceted investigation of the immune-inflammatory-endocrine-metabolic responses to pulmonary tuberculosis and suggested that alterations in the communication between the immune and neuro-endocrine systems in tuberculosis may contribute to disease worsening. del Rey et al. (2007) looked at the interconnectedness of inflammatory cytokines produced during human TB and the endocrine hypothalamic-pituitary-adrenal, -gonadal, and –thyroid systems that could in turn affect the course of infectious/inflammatory processes and or themselves be affected. They proposed that the perpetuation of the lung injury and the hypercatabolic condition that characterises TB disease is lead by the endocrine profiles of TB patients, favouring a reduction of the protective CMI and a worsening of inflammation (del Rey et al. 2007). The review by Bottasso et al. (2010) investigated the underlying interactions of the immune-endocrine-metabolic unit during human TB and concluded that the altered immune-endocrine communication during a hosts' response to TB is affecting essential biological functions. This includes aspects implied in a poorer disease course such as effects on the development of protective responses, control of metabolism, and control of tissue damage (Bottasso et al. 2010). In light of the above and since the current study investigated aspects of the immune/inflammatory, energy metabolism, and the reproductive endocrine systems in lions independently, some insights might be gained by looking at all these systems simultaneously. In order to introduce this topic, I will first give a brief summary of the main research findings in the previous chapters after which a multifaceted investigation will be done and discussed.

6.1.1 Summary of Chapter 1: Introduction

The African lion (*Panthera leo*) population is classified as vulnerable under the IUCN Red List of Threatened Species and in addition to the anthropogenic threats, infectious disease can also contribute to the decline of wild lion populations. One such disease is tuberculosis (TB) caused by *Mycobacterium bovis,* which can cause death in affected lions. A variety of publications report pathology in lions (Cleaveland et al. 2005; Keet et al. 2009; Keet et al. 1996; Keet et al. 2000; Keet et al. 2010; Kirberger et al. 2006; Morris et al. 1996; Trinkel et al. 2011), however, not much has been published on how TB affects the physiological processes ultimately leading to death or the morbid emaciated state of diseased lions. Furthermore, consensus has not been reached on whether TB has an overall negative effect on lion populations. Reaching conclusions on this is further confounded by the chronic nature of the disease, the fact that symptoms only appear during the later stages of disease, and that antemortem diagnostics of *M. bovis* infections in lions are not yet sensitive or specific enough. We are also currently unable to definitively differentiate between exposure, latent disease and active disease in lions.

There is therefore a need, amongst others, for studies that give insights into the processes active or affected during sub-clinical *M. bovis* infections and disease. Such studies would contribute to a knowledge base that can aid in disease management programs and conservation efforts for lions, which are currently largely based on assumptions drawn from other animal models.

6.1.2 Summary of Chapter 2: *M. bovis* diagnostics

In order to conduct antemortem studies, proper diagnostic tests are needed. Various tests have been investigated in this study, such as culture diagnosis using bronchoalveolar lavage (BAL) samples, blood based TB Stat-Pak and QFT gene expression (GEA) assays, and the intradermal tuberculin skin test (ITT).

For some of these diagnostic tests it was possible to identify a similar prevalence of possible *M. bovis* infection in the Kruger National Park (KNP) lion population to that previously reported in other studies (Keet et al. 2000; Sylvester et al. 2016). However, the finding of a relatively high prevalence of possible infection in captive lion collections (presumed to be unexposed to *M. bovis*), illustrates the problems concerning the specificity of some of these tests. For this reason, stringent selection criteria were proposed and using the results of the diagnostic tests two subsets of lions were selected from all the lions to represent two infection categories and used as such in subsequent work. These categories are lions with a high probability of infection ("probably infected", n = 20) and lions unlikely to be infected ("probably uninfected", n = 13).

6.1.3 Summary of Chapter 3: Immune/inflammatory system

During an immune/inflammatory response to a disease/infection, the production of a variety of biomarkers may be stimulated or inhibited. This stimulation or inhibition may differ between type of infection/disease and during the different stages of the immune response to infection/disease

and provide insights into the different types of immune responses present, or perhaps indicate whether an animal is experiencing disease-related stress, or even be useful as diagnostic aids (Chegou et al. 2016; Essone et al. 2014; Fearon & Locksley, 1996; Jacobs et al. 2016a; Jacobs et al. 2016c; Jacobs et al. 2016b; Pollock et al. 2001; Rook, 1999; Santucci et al. 2011; Sutherland et al. 2016).

The biological markers investigated in the current study were CRP, cortisol, and an array of cytokines. Very little data have been previously published on the normal ranges of these markers in lions.

Results from the current study serve as an indication that the KNP lion population, when compared to captive lions, was experiencing Th-1 and/or Th-2 immune responses with elevated stress and inflammation. Since infection status was not confirmed with necropsy it was not possible to provide a definitive conclusion that *M. bovis* infection is the sole reason for the observed differences between the wild/*M. bovis* exposed and captive/*M. bovis* unexposed lions. The same can be said for the differences observed between the "probably infected" and "uninfected" lion groups.

6.1.4 Summary of Chapter 4: Energy metabolism

With the advanced stages of TB in lions associated with clinical manifestations such as emaciation and the strong link between human TB cases and energy imbalance, an investigation of how/if lion energy metabolism is affected by *M. bovis* infection/disease was considered for the current study.

The biological markers used for this investigation were glucose, insulin, leptin, ghrelin, and %HbA1c. Results from the current study tentatively suggest that some form of altered energy metabolism might be present in either the captive or wild lion populations. Uncertainty as to whether the energy metabolism alterations are due to *M. bovis* in the wild lions or due to housing and feeding practices in the captive lions was caused by many confounding factors such as a lack of assays specific to biological markers of lions, the possible effect of the anaesthetic drugs on the circulating concentrations of the markers such as glucose and insulin, and the inability to regulate feeding behaviour of the wild lions.

6.1.5 Summary of Chapter 5: Reproductive endocrinology

The reproductive endocrine system consists of a cascade of neural pathways, endocrine glands, and hormones. Normal reproductive functioning of organisms is maintained through various positive and negative hormonal feedback loops as well as various external inhibitors and stimulants. In this regard altered energy metabolism and/or immune system function can negatively affect an individuals' ability to reproduce. In order to gain insights into the current state of a lions' reproductive system it is possible to measure the levels of reproductive hormones such as testosterone, oestradiol, and progesterone in blood or faecal samples. However, single point samples are not ideal to describe in detail the functioning of an individual lions' reproductive neuro-

endocrine system since the natural concentrations of hormones are dynamic and can fluctuate within minutes. One way to get around this problem is to do provocative tests with repeat samples taken over an extended period of time.

The current study made use of both single point sampling and a provocative test to investigate possible effects of *M. bovis* on the reproductive neuro-endocrine system of lions. Differences were observed in testosterone concentrations between wild and captive lions. However, the possibility exists that these differences are artefacts due the hormonal assays or due to effects of the anaesthetic drugs used. Limitations with the available hormonal assays hampered the ability to ascertain if *M. bovis* is affecting the female reproductive neuro-endocrine system.

Previous studies (Brown et al. 1991; Brown et al. 1993) investigated the pituitary functioning of lions by means of a GnRH challenge. The current study was the first to show that the Kisspeptin system (situated higher in the reproductive cascade than GnRH) can be used to investigate lion reproductive neuro-endocrine functioning.

With all of the above in mind, the following section and aim of this chapter is to investigate possible interactions between the different systems investigated in the current study.

6.2 Materials and Methods

In order to search for possible interactions between the immune/inflammatory, the energy (metabolic), and the reproductive endocrine systems of lions in the current study, all biological markers for which data were available were considered. Additionally, quantitative diagnostic data generated by means of the QFT GEA (Chapter 2) were also included in the investigation. Since testosterone was only measured in male lions it was excluded from the initial analyses of all the lions and the subset of probably infected and uninfected lions. The main cohorts studied in this chapter were the presumed *M. bovis* exposed and unexposed cohorts as well as the probably *M. bovis* infected and uninfected cohorts (See Chapter 3 section 3.2.1 for more detail on the cohort selection criteria).An additional investigation was done for male lions only, where testosterone concentrations were included in the statistical analyses.

6.2.1 Statistical analyses

Multivariate statistical analyses were done with R using RStudio for Windows (version 0.99.482) (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, http://www.rstudio.com/).

As described in Chapter 3 sections 3.3.3 and 3.4.2, some of the lions had cytokine values considered to be outliers and one lion had an outlier insulin value (see Chapter 4 section 4.3.4). Statistical analyses were done first with all the data from all the lions (including outliers) after which lions that had outlier cytokine and insulin values were excluded from the analyses. As described in Chapter 3 section 3.3.3, outlier cytokine values in probably infected lions could have been due to the presence of infection or disease and excluding these animals could result in the exclusion of

cytokine values able to describe altered immune states in the presence of *M. bovis* infection/disease. Therefore, lions included in the probably infected and uninfected subsets were not excluded when outlier cytokine values were excluded. Some lions did not have QFT GEA values and had to be excluded from the multivariate statistical analyses. This had to be done since the multivariate statistical models and software used to do the calculations are not able to work with data frames that have missing data points.

Scatterplot-matrices were compiled for the different markers. Due to the large size of these plots, only those markers that showed relatively high Spearman's correlation coefficients will be shown in the results section. Principle component analyses (PCA) were done with data normalised by means of the "scale" function in R with the formula: normalised x = (x – column mean)/column standard deviation. The PCA results were plotted as score and correlation plots. The different categorical variables were respectively super-imposed on the PCA plots. In some instances, distance based cluster analyses were done using all or a selection of the biological markers.

Packages used in R:

- "base" package – PCA and cluster analyses
- "ggplot" package – Plotting of PCA score and correlation plots
- "car" package – Scatterplot-matrix and spearman correlations

On the occasions where finer scale correlations were inspected, GraphPad Prism Version 4 for Windows (GraphPad Software, Inc., CA, USA) was used. Correlations were done using the non-parametric Spearman's correlation test.

6.3 Results

6.3.1 Multivariate analyses with data from all the sampled lions

Figures 6.1 and 6.2 shows excerpts from the scatterplot matrices generated with the biological markers of all the lions sampled in this study with cytokine and insulin outlier values included and excluded respectively. Due to the large plot size when all the biological markers are used, only the markers that had Spearman's correlation coefficients ≥ 0.5 with at least one other marker were included in the excerpts. Excluding the lions with cytokine and insulin outliers resulted in the QFT GEA marker being included in the selection of markers that showed a higher Spearman's correlation. The Spearman's correlation between insulin and CRP (Spearman's r = 0.57, $p < 0.0001$) were constant despite the removal of lions with outlier cytokine and insulin values. A relatively strong Spearman's correlation can be seen between cortisol and the QFT GEA values with the linear regression showing increased cortisol concentrations associated with higher GEA values (Figure 6.2).

Figure 6.1: Scatterplot matrix excerpt of a selection of biological markers that had a Spearman's correlation coefficient greater than 0.5 with at least one other marker. Data from all lions sampled in this study used (including lions with outlier cytokine or insulin values). Linear regression and Spearman's correlations between the different markers are indicated.

Figure 6.2: Scatterplot matrix excerpt of a selection of biological markers that had a Spearman's correlation coefficient greater than 0.5 with at least one other marker. Data of all lions sampled in this study used (Excluding lions that had outlier cytokine or insulin values). Linear regression and Spearman's correlations between the different markers are indicated.

The PCA results presented in Table 6.1 show that a greater proportion of the variance could be explained by the first principle component (PC1-2) when the lions with outlier cytokine and insulin values were excluded from the analyses (23% with outliers excluded compared to 18% with outliers included). With outliers excluded, cortisol, CRP and glucose concentrations contributed the most to PC1-2 while IL-8, IL-10 and insulin contributed the most to the second principle component (PC2-2). Using the data from all the lions (Figure 6.3) the *M. bovis* unexposed lions associated more with higher glucose values while the *M. bovis* exposed lions associated more with increased cortisol, CRP and IP-10 values. When the lions with outlier cytokine and insulin values were excluded from the analyses (Figure 6.4), the above mentioned associations remained while the separation of the two exposure groupings became more pronounced.

Table 6.1: Summary of the PCA results for the first two components of all the biological markers for all the captured lions. Values in bold indicate the biological markers that contribute the most to the relevant principle component. (PC1-1 and PC2-1 = Principle components one and two of the biological markers for all the lions; PC1-2 and PC2-2 = Principle components one and two for the biological markers of all the lions excluding the specified cytokine and insulin outliers)

	All*		Excluding outliers[#]	
	PC1-1	**PC2-1**	**PC1-2**	**PC2-2**
Proportion of variance	0.18	0.17	0.23	0.14
Insulin	0.22	-0.15	0.25	-0.09
Glucose	**-0.43**	0.10	**-0.41**	0.01
Leptin	-0.16	0.11	-0.14	**0.37**
Ghrelin	0.05	**-0.46**	0.12	-0.05
BMI	-0.29	0.09	-0.24	0.34
QFT GEA[$]	0.12	-0.22	0.19	0.36
KC-Like	-0.20	**-0.42**	-0.10	0.24
IP-10	**0.38**	0.03	0.34	-0.01
IL-8	-0.10	**-0.44**	-0.22	**-0.50**
IL-10	0.08	-0.30	-0.15	**-0.46**
TNF-a	0.14	**0.45**	0.25	-0.25
VEGF	-0.14	-0.12	-0.12	0.11
Cortisol	**0.48**	0.04	**0.44**	0.05
CRP	**0.41**	-0.10	**0.41**	0.00

*PCA results using all biological markers measured in this study, excluding testosterone, for all the lions sampled.

[#]Lions that had outlier cytokine values and were not included in the probably infected and uninfected subsets were excluded. One lion with an outlier insulin value was also excluded.

[$]The quantitative QFT GEA data generated for the diagnosis of *M. bovis* infections in Chapter 2 was also included in the PCA analyses since this diagnostic test makes use of immune response markers.

Figure 6.3: Score and correlation plot with the data from all lions sampled in the current study (cytokine and insulin outliers included). Different colours indicate different *M. bovis* exposure groups.
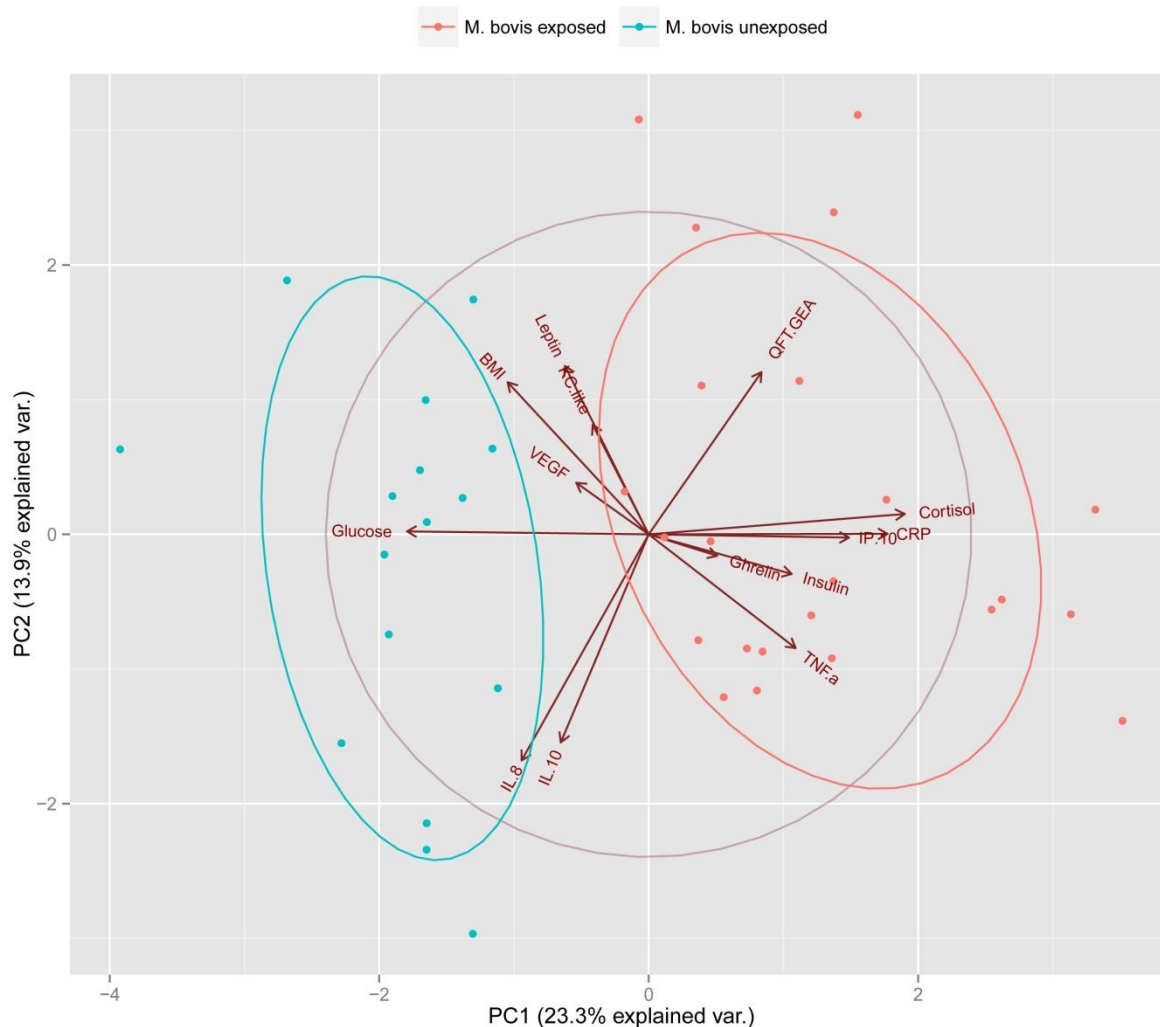
Figure 6.4: Score and correlation plot with the data from all lions sampled in the current study (cytokine and insulin outliers excluded). Different colours indicate different *M. bovis* exposure groups.

6.3.2 Multivariate analyses using biological markers of the lions included in the probably infected and uninfected subsets

Figure 6.5 is an excerpt of the scatterplot matrix of all the biological markers for the lions categorised according to the probable infection status. Only biological markers that had a Spearman's correlation coefficient of ≥ 0.5 with at least one other marker are included in this plot. The effect of the insulin outlier can be seen in the far left column of Figure 6.5 and a scatterplot matrix with this lion excluded was compiled with the excerpt of this plot shown in Figure 6.6. Regardless of the inclusion or exclusion of the lion with the outlier insulin value, CRP had relatively high Spearman's correlations with more of the other biological markers than had any of the other markers. A relatively strong correlation (Spearman's r = 0.56, p = 0.0013) can be seen between CRP and BMI with higher CRP concentrations associated with lower BMI scores. A similar correlation can be seen between CRP and insulin (Spearman's r = 0.52, p = 0.0022) with increased insulin concentrations associated with increased CRP. The strongest correlation

observed is between cortisol and the quantitative data of the QFT GEA (Spearman's r = 0.75, p < 0.0001, Figure 6.6) indicating that lions with higher GEA values (and therefore more likely to be *M. bovis* infected) had higher circulating cortisol concentrations.
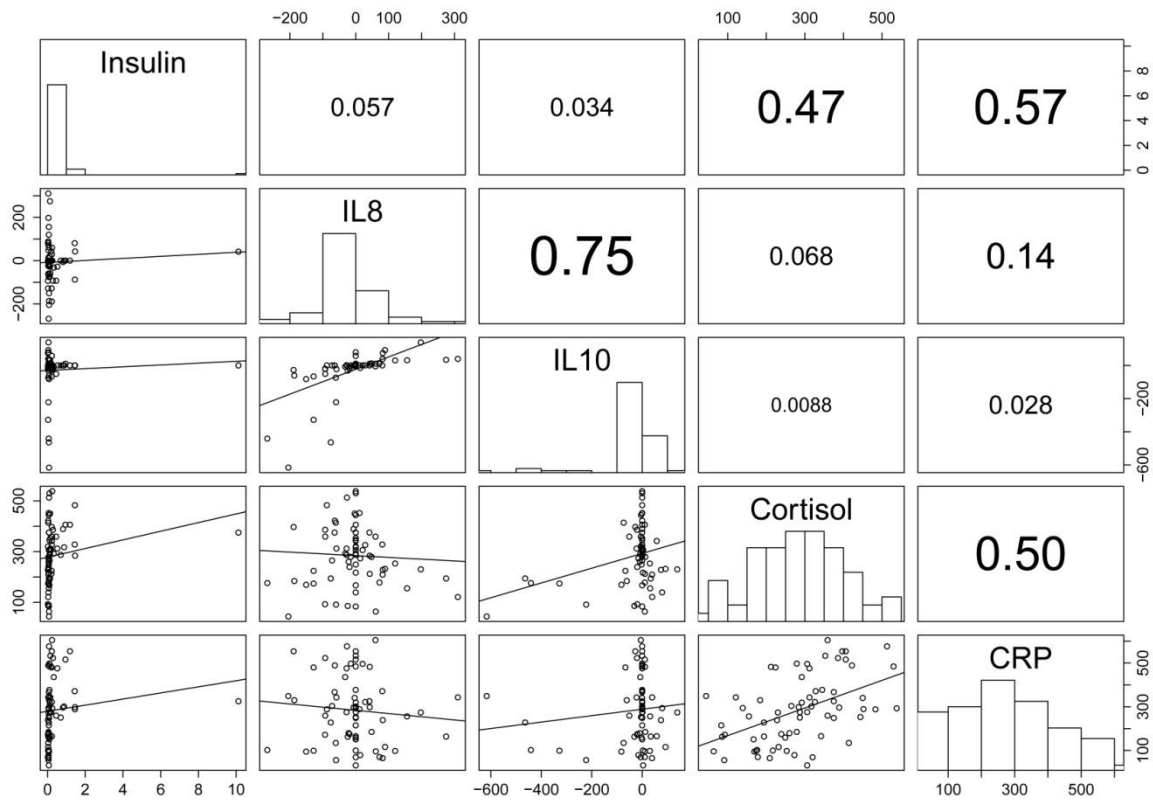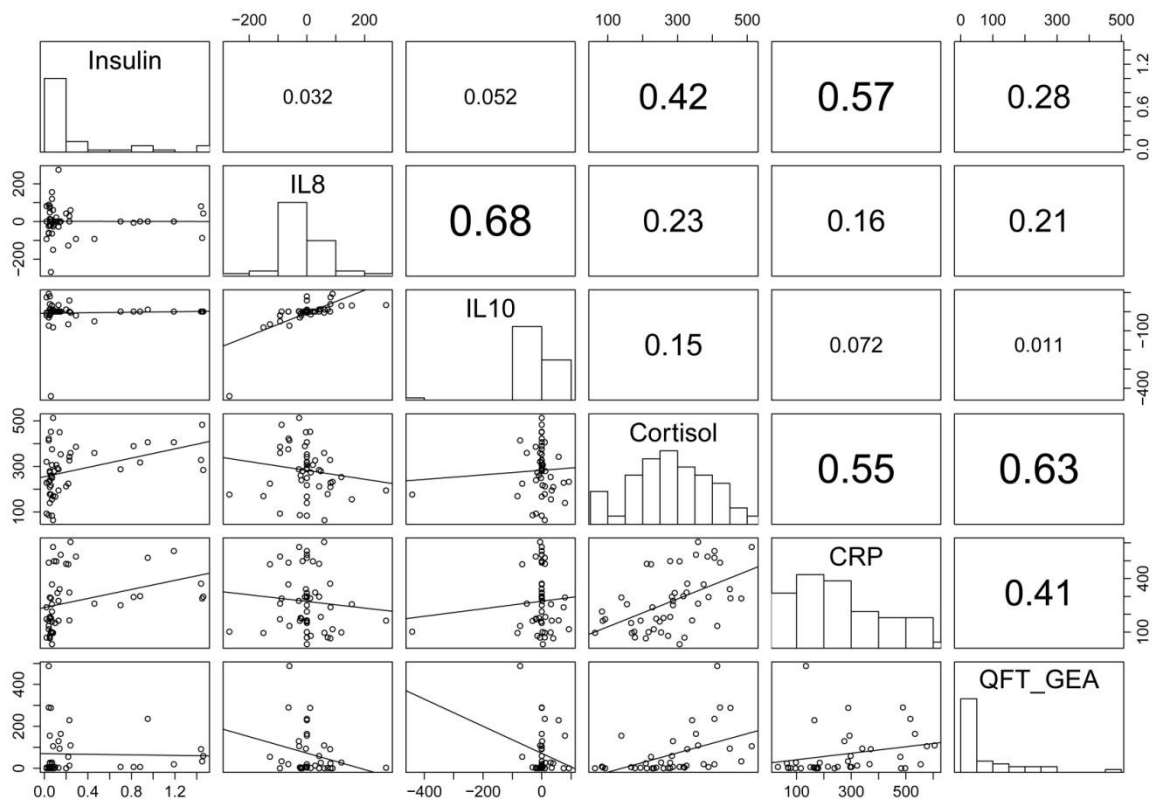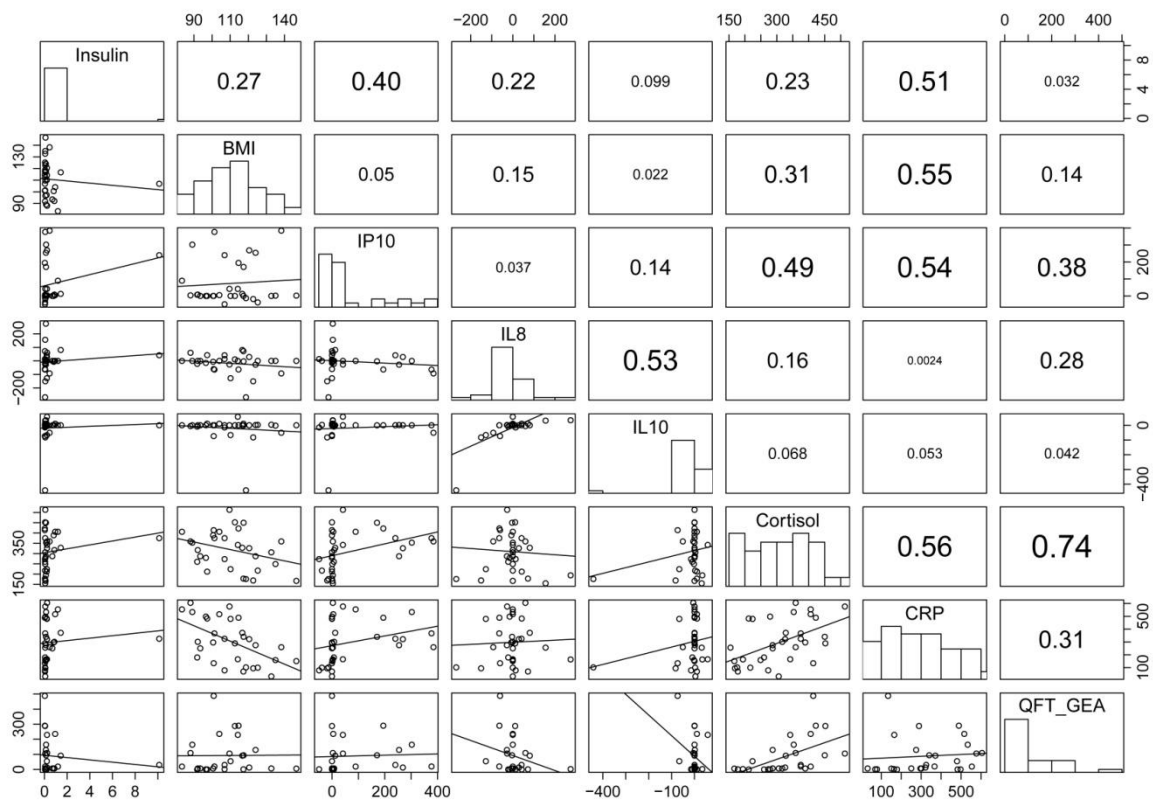

Figure 6.5: Scatterplot matrix excerpt of a selection of biological markers that had a Spearman's correlation coefficient greater than 0.5 with at least one other marker. Data of lions included in the probably infected and uninfected subsets were used (Lion with outlier insulin value included). Linear regression and Spearman's correlations between the different markers are indicated.
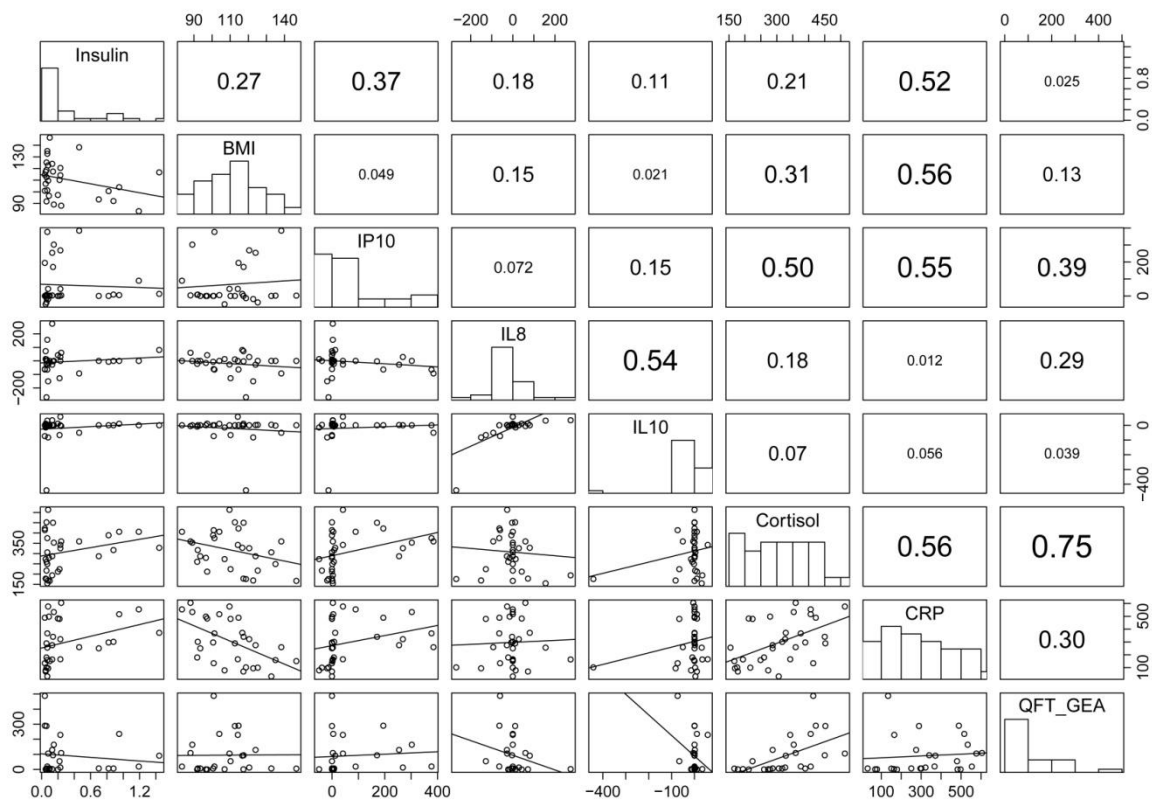
Figure 6.6: Scatterplot matrix excerpt of a selection of biological markers that had a Spearman's correlation coefficient greater than 0.5 with at least one other marker. Data of lions included in the probably infected and uninfected subsets were used (Lion with outlier insulin value excluded). Linear regression and Spearman's correlations between the different markers are indicated.

PCA's were done with the data of the biological markers measured in the lions classified as probably infected and uninfected. A summary of the results for the first two principle components can be seen in Table 6.2. When the lion with the outlier insulin concentration was included in the analyses, PC1-1 explained 23% of the variance in the data set with glucose and leptin contributing the most to PC1-1 and the QFT GEA and cortisol values the most to the PC2-1. Exclusion of the lion with the outlier insulin concentration resulted in PC1-2 explaining 22% of the variance and BMI and CRP were included with glucose and leptin as the major contributors to PC1-2. The score and correlation plots for these analyses (Figures 6.7 and 6.8) show some overlap between the probably infected and uninfected lions. In both cases (outlier included and excluded) differentiation between probably infected and uninfected lions is more pronounced along the PC2 axis (Figures 6.7 and 6.8) with probably uninfected lions associating more with higher IL-8 and IL-10 values while probably infected lions associate more with higher QFT GEA values and increased cortisol concentrations.

Table 6.2: Summary of the PCA results for the first two components of all the biological markers for the lions included in the probably infected and uninfected subsets. Values in bold indicate the biological markers that contribute the most to the relevant principle component. (PC1-1 and PC2-1 = Principle components one and two for the biological markers of the probably infected and uninfected lions; PC1-2 and PC2-2 = Principle components one and two for the biological markers of probably infected and uninfected lions excluding the insulin outlier)

	Subset*		Excluding outlier[#]	
	PC1-1	**PC2-1**	**PC1-2**	**PC2-2**
Proportion of variance	0.23	0.16	0.22	0.16
Insulin	0.22	-0.15	0.12	-0.06
Glucose	**-0.37**	0.01	**-0.38**	0.07
Leptin	**-0.35**	-0.06	**-0.37**	0.03
Ghrelin	0.26	-0.11	0.17	-0.02
BMI	-0.31	-0.15	**-0.37**	-0.04
QFT GEA[$]	-0.06	**0.52**	-0.01	**0.56**
KC-Like	-0.27	0.32	-0.25	0.39
IP-10	0.31	0.04	0.28	0.07
IL-8	0.10	-0.39	0.03	-0.37
IL-10	0.11	-0.28	0.08	-0.28
TNF-a	0.32	0.10	0.35	0.03
VEGF	-0.27	0.18	-0.23	0.23
Cortisol	0.24	**0.47**	0.28	**0.46**
CRP	0.30	0.26	**0.37**	0.18

*PCA results using all biological markers measured in this study, excluding testosterone, for the lions included in the probably infected and uninfected subsets.

[#]One lion with an outlier insulin value was excluded.

[$]The quantitative QFT GEA data generated for the diagnosis of *M. bovis* infections in Chapter 2 was also included in the PCA analyses since this diagnostic test makes use of immune response markers.
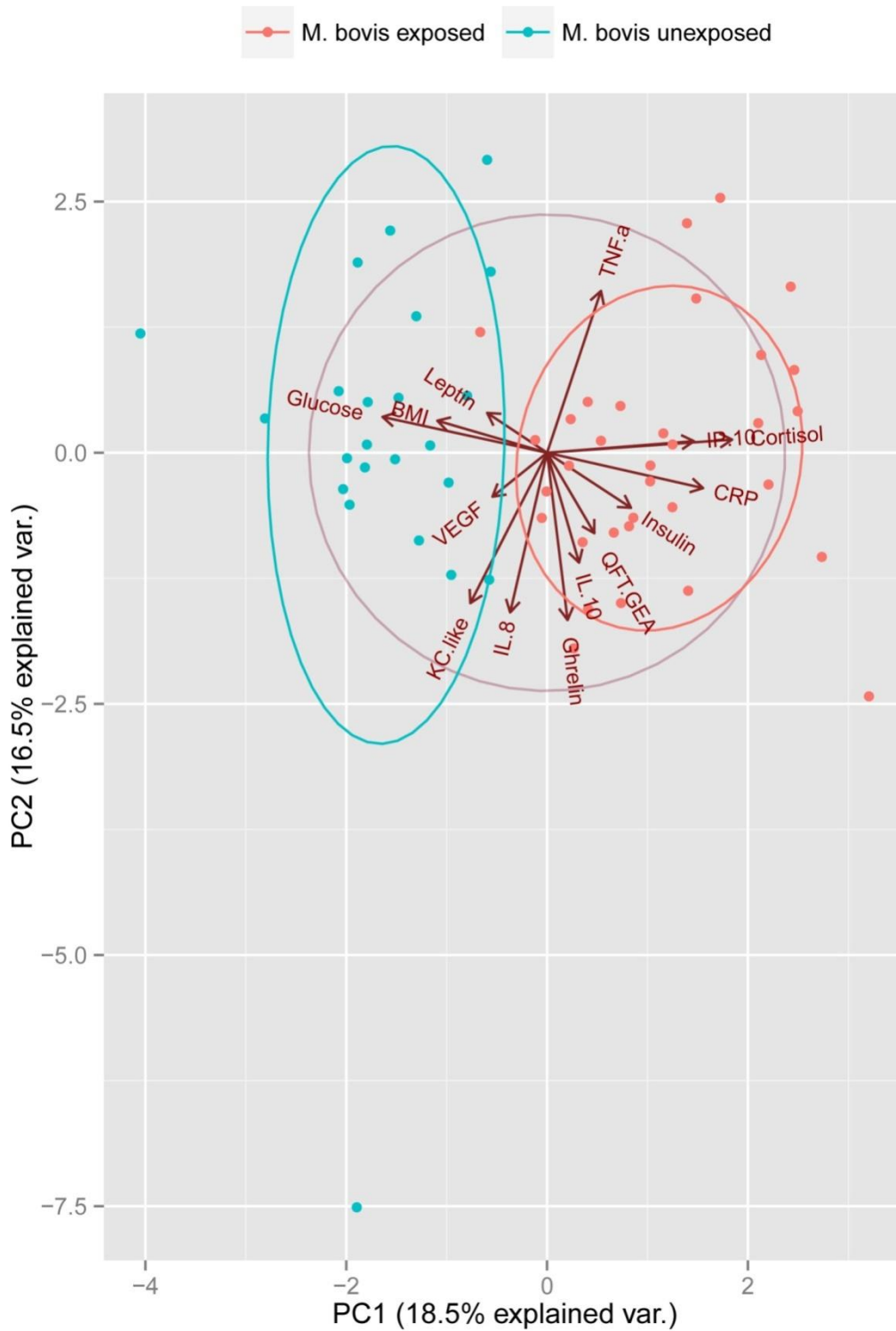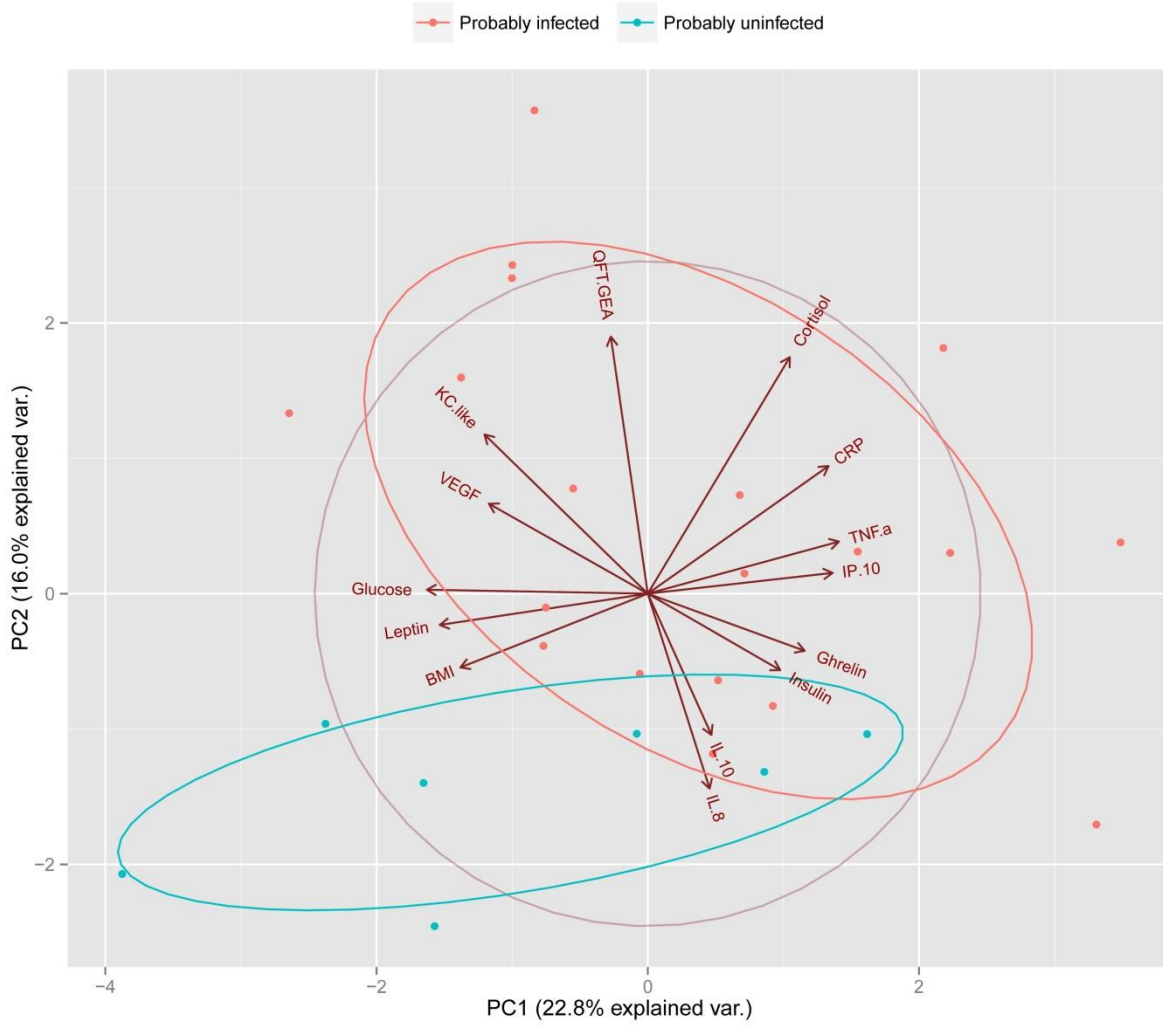
Figure 6.7: Score and correlation plot with the data from all lions included in the probably infected and uninfected subsets (insulin outlier included). Different colours indicate probability of infection status.
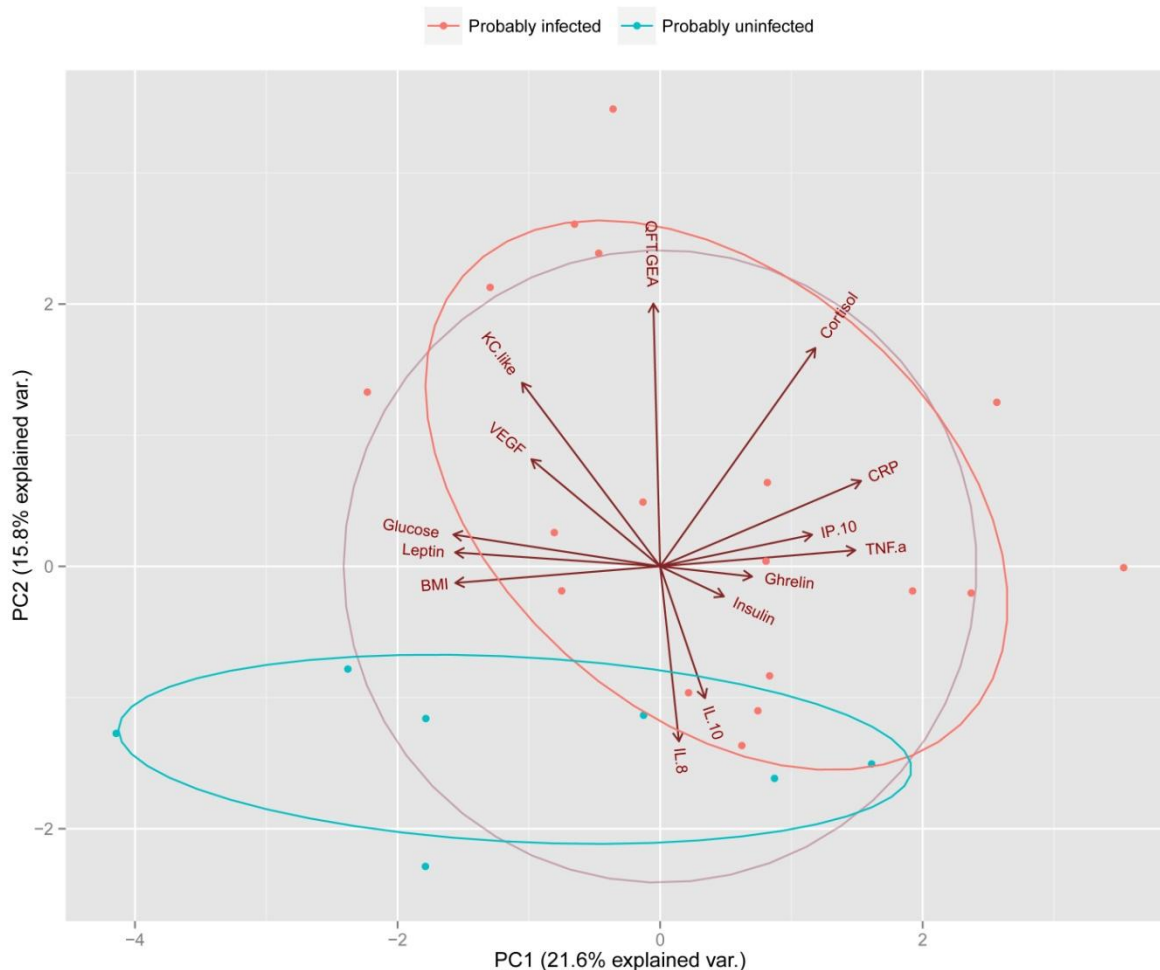
Figure 6.8: Score and correlation plot with the data from all lions included in the probably infected and uninfected subsets (insulin outlier excluded). Different colours indicate probability of infection status.

Cluster analyses were also attempted with the biological markers of the lions classified as probably infected and uninfected. With this analysis it was not possible to properly differentiate between probably infected and uninfected lions (Figure 6.9). An attempt was made to do a more focussed cluster analysis using a selection of biological markers (Figure 6.10). This selection comprised of markers identified by means of the scatterplot matrix (Figure 6.6) and the PCA (Table 6.2) to most likely play a role in the differentiation between the probably infected and uninfected subsets. With this selection of markers it was not possible to differentiate between probably infected and uninfected lions by means of cluster analysis.
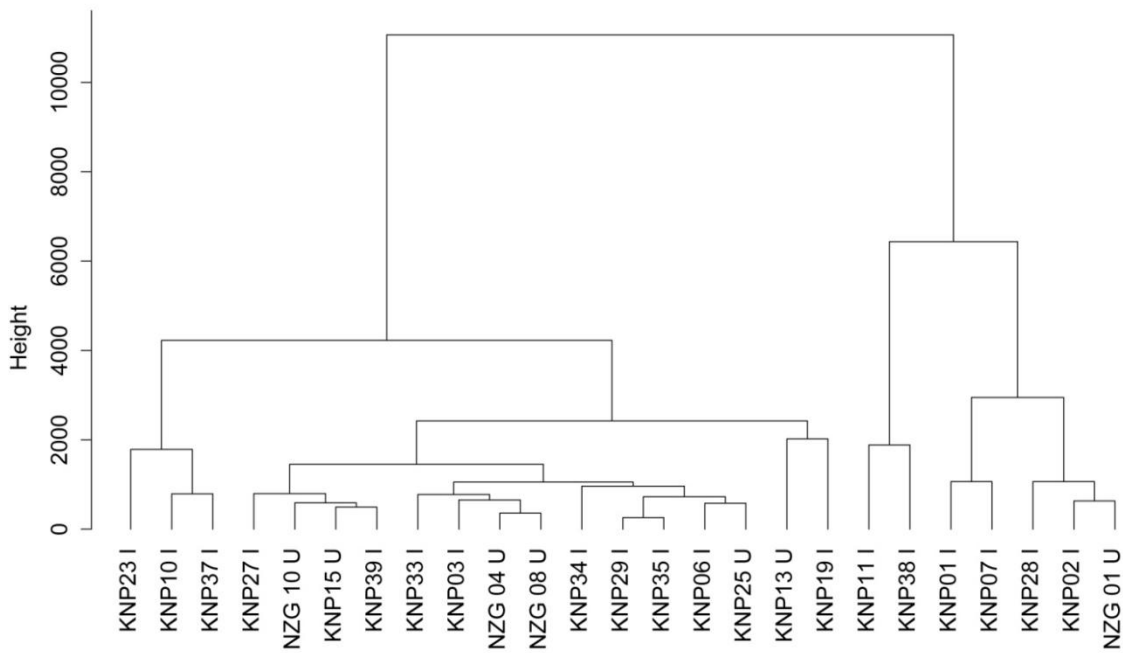
Figure 6.9: Cluster analyses dendrogram for all the biological markers of the lions classified as probably infected and uninfected. (I = probably infected, U = probably uninfected)
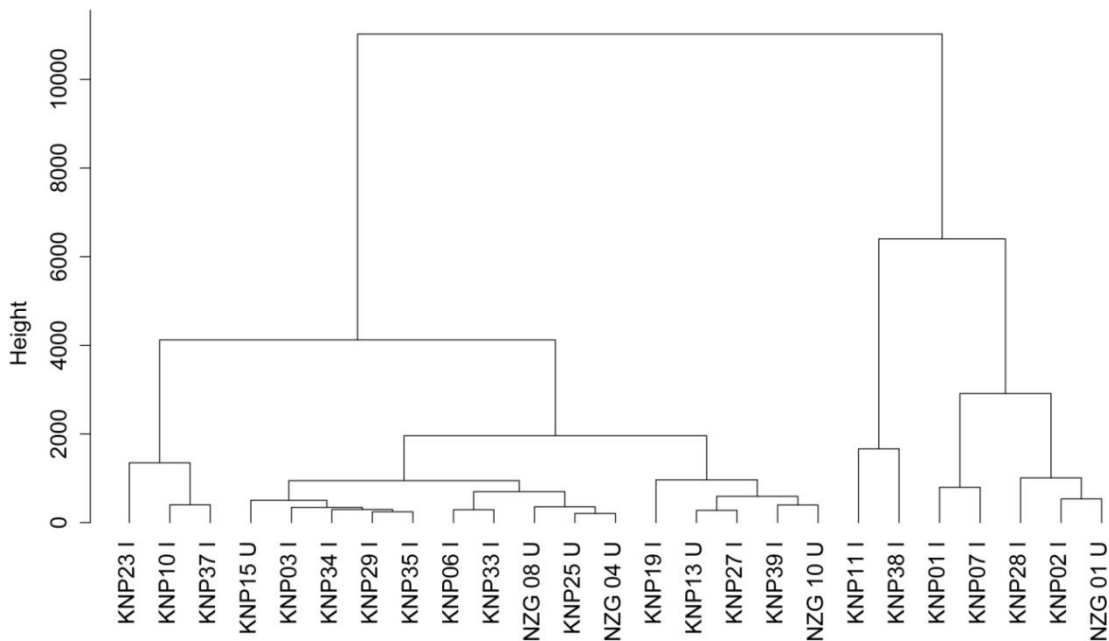
Figure 6.10: Cluster analyses dendrogram compiled with a selection of biological markers (Glucose, leptin, BMI, QFT GEA, KC-Like, IP-10, IL-8, cortisol, and CRP) of the lions classified as probably infected and uninfected. (I = probably infected, U = probably uninfected)

6.3.3 Investigation with reproductive hormone data included

From the results described in Chapter 5 it was possible to get meaningful data for only the male reproductive hormone testosterone. Therefore analyses with all the biological markers measured in this study, including testosterone, were done for only the male lions. An excerpt of the

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scatterplot matrix drawn with this data can be seen in Figure 6.11. Only biological markers that had Spearman's correlation coefficients of ≥ 0.5 with at least one other marker are included in the excerpt. For the males, the greatest correlation (Spearman's $r = 0.86$, $p < 0.0001$) is seen between testosterone concentrations and BMI with increased BMI associated with increased testosterone. A high correlation can also be seen between testosterone and CRP (Spearman's $r = 0.65$, $p = 0.0004$) with increased CRP concentrations associated with decreased testosterone concentrations. A Similar situation can be seen between testosterone and insulin (Spearman's $r = 0.6$, $p = 0.0014$) where increased insulin concentrations are associated with decreased testosterone. Increased insulin was associated with increased cortisol (Spearman's $r = 0.59$, $p = 0.0020$) and increased CRP (Spearman's $r = 0.63$, $p = 0.0007$). Lastly an increased BMI was associated with decreased CRP concentrations (Spearman's $r = 0.57$, $p = 0.0031$). The relatively strong positive correlations of testosterone with BMI and insulin with CRP can also be observed in the PCA score and correlation plots of the same data presented in Figures 6.12, 6.13, and 6.14.
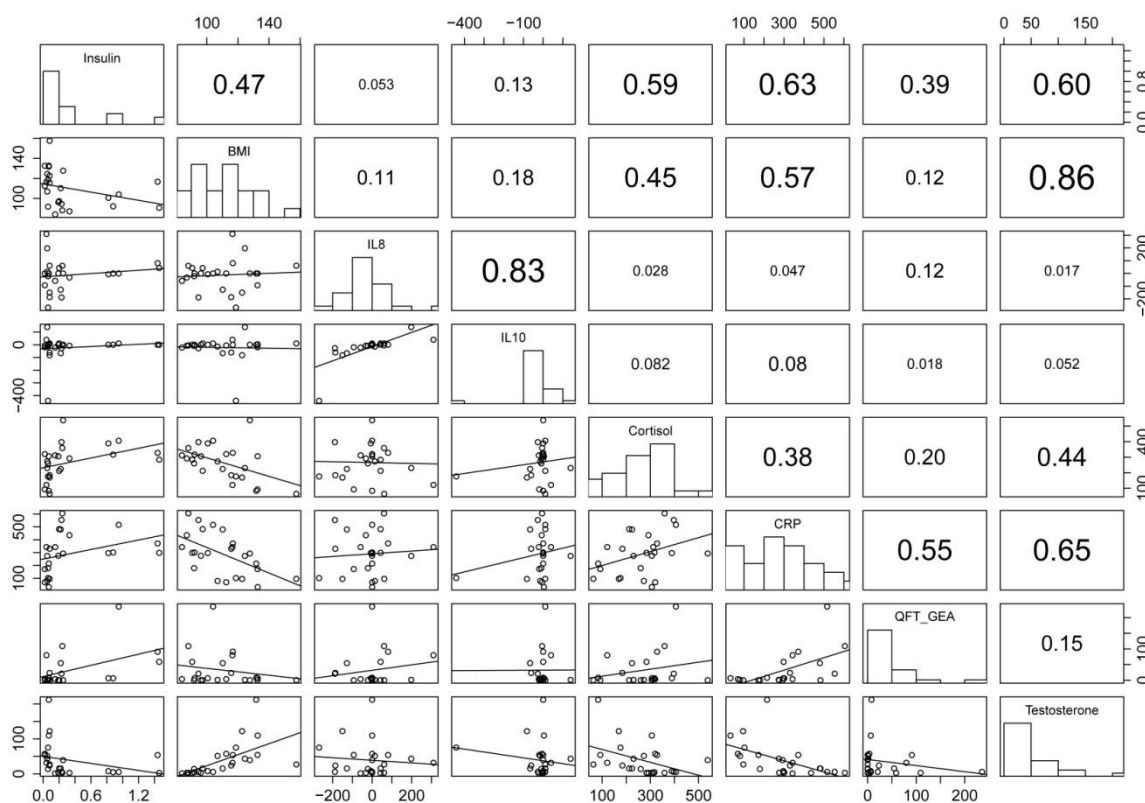
Figure 6.11: Scatterplot matrix excerpt of a selection of biological markers that had a Spearman's correlation coefficient greater than 0.5 with at least one other marker. Only data of male lions were used (Males with outlier cytokine values were included). Linear regression and Spearman's correlations between the different markers are indicated.

A summary of the PCA results using all biological markers measured in male lions in the present study can be seen in Table 6.3. PC1 and PC2 explained similar proportions of the variance in the data set with respectively 22% and 21%. The biological markers that contributed the most to PC1 were Insulin, BMI, QFT GEA, and CRP while Ghrelin, IL-8, and TNF-α contributed the most to PC2.

The score and correlation plot of this PCA was inspected by super-imposing the categorical variables of KNP age class (Figure 6.12), *M. bovis* exposure (Figure 6.13), and probability of *M. bovis* infection (Figure 6.14) over it. Figure 6.12 shows that much of the differentiation between the age classes occur along the PC1 axis with sub-adult males overlapping more with adult males than with juvenile males. Some overlap occurs between adult and juvenile males. In general juvenile males associate more with higher insulin and CRP concentrations while adult males associate more with higher BMI scores and increased testosterone and leptin concentrations.

In Figure 6.13 a clear differentiation can be observed between *M. bovis* exposed and unexposed male lions. *M. bovis* unexposed lions associate more with higher BMI scores and higher testosterone and leptin concentrations while the *M. bovis* exposed lions show a greater association with increased cortisol, insulin, and CRP concentrations.

When the categorical variable of probable *M. bovis* infection status is superimposed on the score and correlation plot (Figure 6.14) a relatively clear differentiation between the probably uninfected and infected lions can be seen with lions that were not included in these subsets overlapping with both of them. The probably uninfected males show an association with increased glucose and leptin concentrations while the probably infected males associate more with increased CRP and insulin concentrations.

Table 6.3: Summary of the PCA results for the first two components of all the biological markers for the male lions. Values in bold indicate the biological markers that contribute the most to the relevant principle component.

	Males*	
	PC1	PC2
Proportion of variance	0.22	0.21
Testosterone	0.33	-0.12
Insulin	**-0.35**	0.04
Glucose	0.25	0.11
Leptin	0.33	0.07
Ghrelin	-0.04	**-0.43**
BMI	**0.39**	-0.14
QFT GEA$	**-0.34**	-0.17
KC-Like	-0.19	**-0.37**
IP-10	-0.06	0.13
IL-8	-0.01	**-0.43**
IL-10	0.04	-0.30
TNF-a	0.04	**0.46**
VEGF	-0.13	-0.02
Cortisol	-0.32	0.30
CRP	**-0.40**	0.02

*PCA results using all biological markers measured in this study for male lions.

$The quantitative QFT GEA data generated for the diagnosis of *M. bovis* infections in Chapter 2 was also included in the PCA analyses since this diagnostic test makes use of immune response markers.
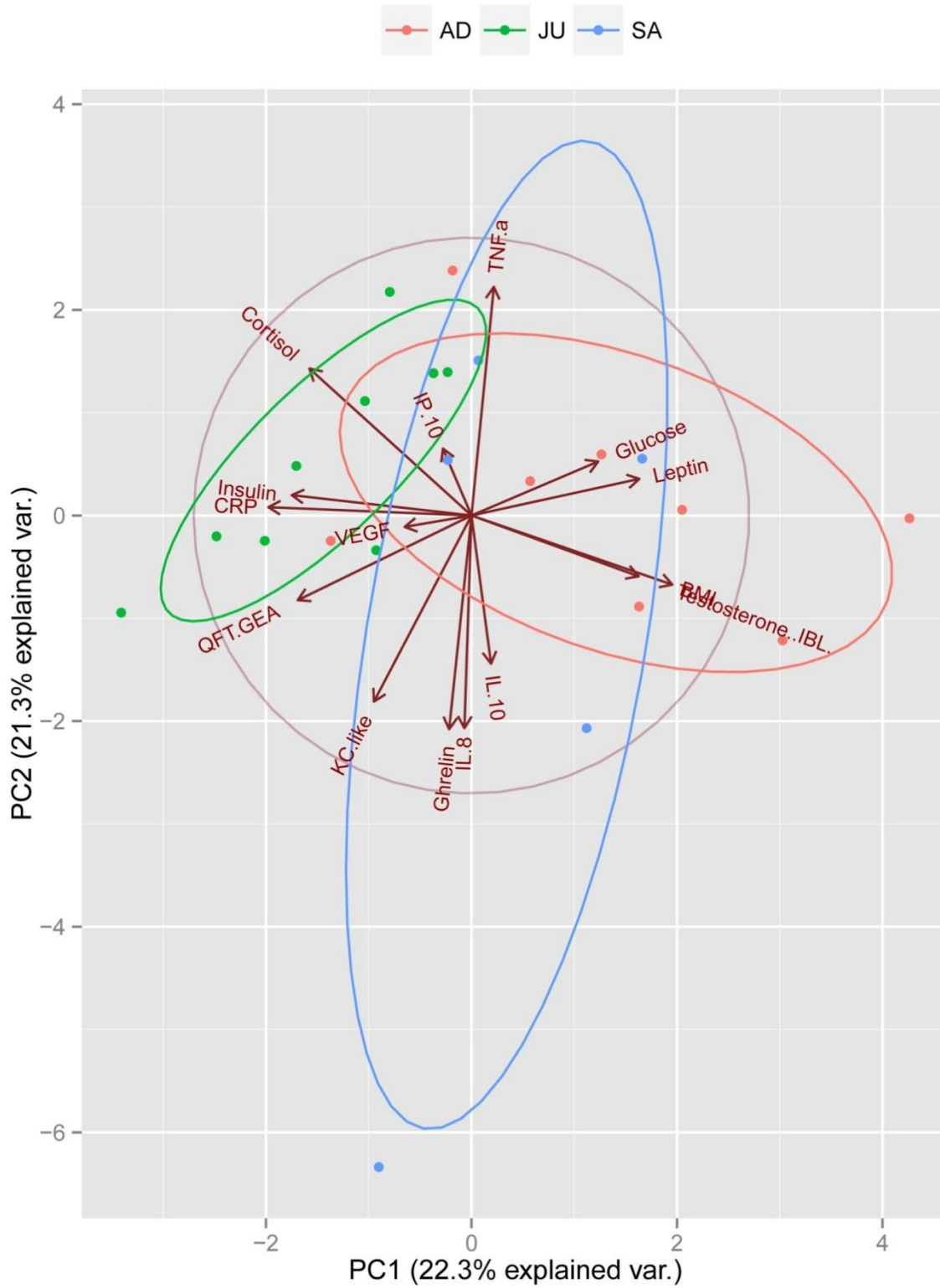
Figure 6.12: Score and correlation plot with the data of male lions (Cytokine outlier values included). Different colours indicate KNP age class (AD=adult, JU=juvenile, SA=Sub-adult).
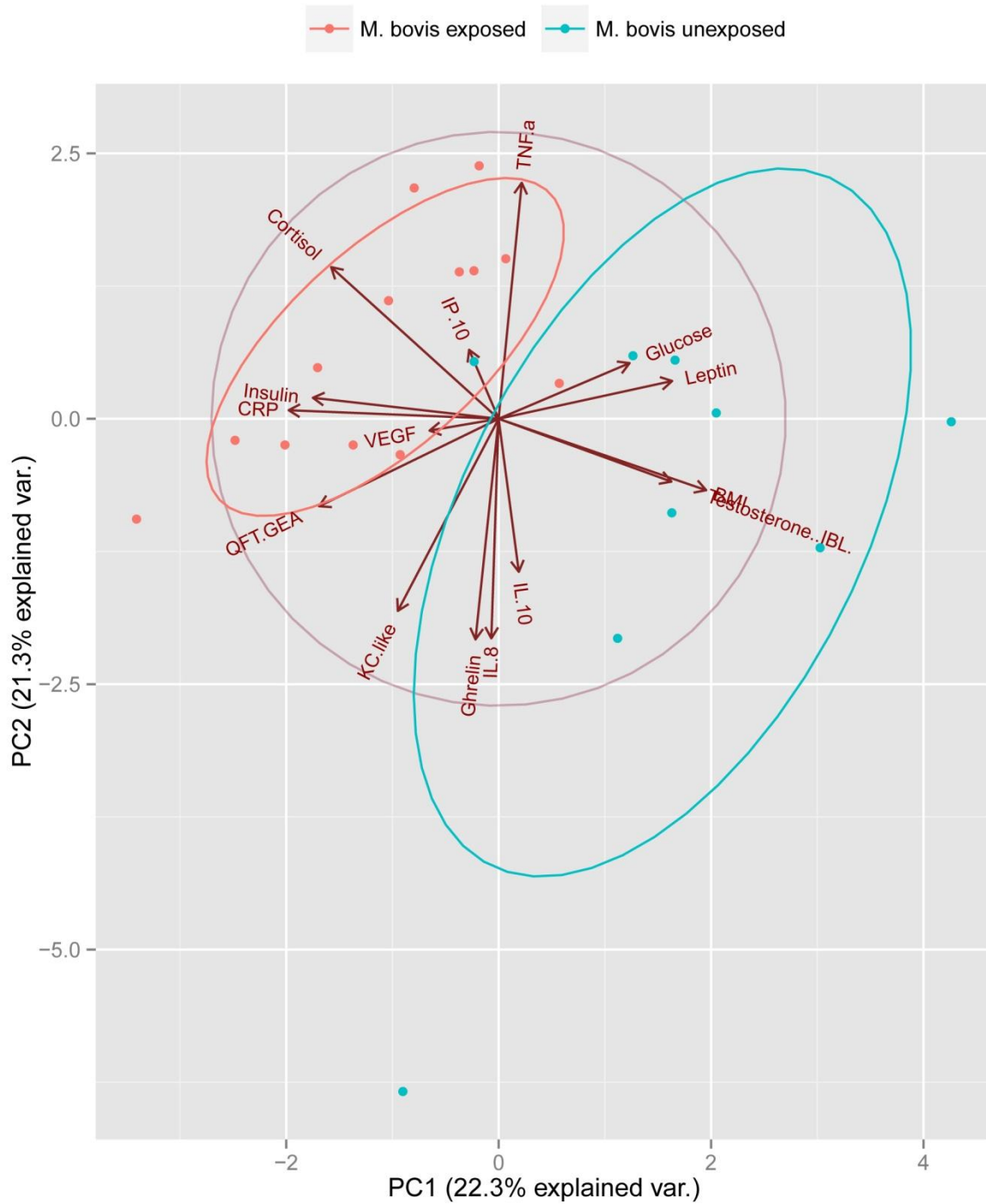
Figure 6.13: Score and correlation plot with the data of male lions (Cytokine outlier values included). Different colours indicate *M. bovis* exposure.
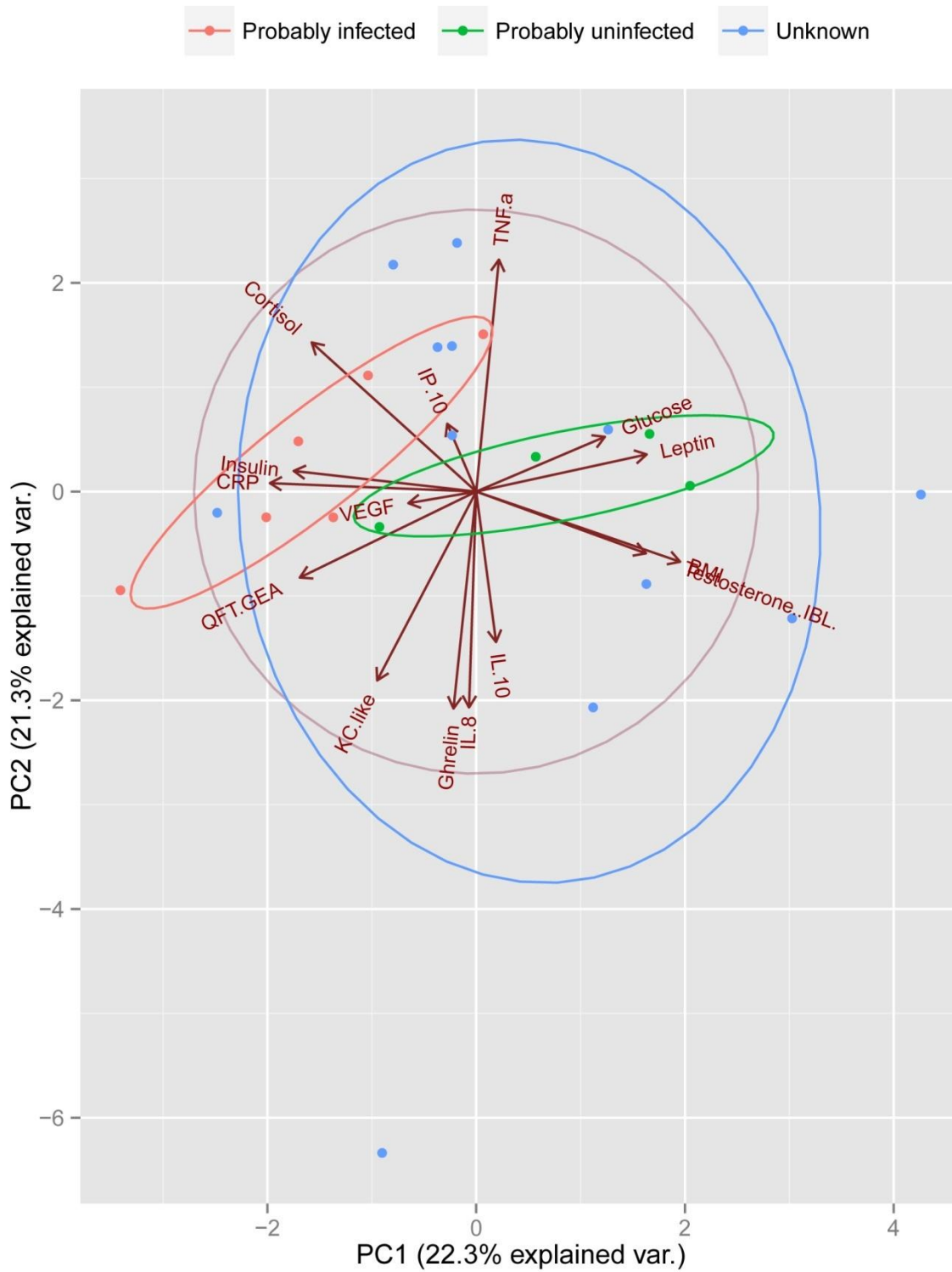
Figure 6.14: Score and correlation plot with the data of male lions (Cytokine outlier values included). Different colours indicate probability of *M. bovis* infection.

6.4 Discussion

The results pertaining to respective immune/inflammation, energy metabolic, and reproductive endocrine systems were discussed separately in their representative chapters. This discussion will be mainly focussed on the interactions observed between the systems.

6.4.1 Cross systems investigation using all the biological markers of all the sampled lions.

Initial inspection was done with the data from all the lions sampled in this study. The PCA results (Table 6.1) and score and correlation plots (Figures 6.3 and 6.4) showed a relatively clear differentiation between the *M. bovis* exposed and unexposed lion populations. Regardless of whether the lions with cytokine and insulin outliers were excluded or included from the analyses, the major contributors to the differentiation along the PC1 axis were glucose, cortisol, and CRP (Table 6.1) where captive/*M. bovis* unexposed lions had higher glucose concentrations (discussed in Chapter 4 section 4.4.1) and lower cortisol and CRP concentrations (discussed in Chapter 3 section 3.4.1) than the wild/*M. bovis* exposed lions.

The scatterplot matrix (Figure 6.1) showed that the only energy metabolism marker that had a Spearman's correlation coefficient of > 0.5 with an immune/inflammation marker was insulin. A significant correlation was observed between insulin and CRP. In humans, insulin resistance is associated with increased levels of the cytokines TNF-α and IL-6 (pro-inflammatory cytokines also produced by adipocytes). These cytokines, in addition to their inhibitory actions on insulin sensitivity, also lead to increased hepatic CRP production (Pradhan et al. 2003; Wilcox, 2005). Note: In the current study increased TNF-α was associated with increased CRP.

Additionally, it has been proposed that an up-regulated inflammatory response can influence hyperinsulinaemia and the development of an insulin-resistant state (Pradhan et al. 2003). Considering this together with the suggestion that insulin resistance might serve as a marker and potential risk factor for active *M. tuberculosis* infection in humans (Mao et al. 2011); that levels of TNF-α are increased in probably infected lions (although significance at p ≤ 0.05 was not found in current study; see Chapter 3 section 3.4.2); that levels of circulating insulin were higher in the probably infected lions (Chapter 4 section 4.4.2); and that probably infected lions had higher concentrations of circulating CRP (Chapter 3 section 3.4.1), the finding that CRP and insulin had a significant positive correlation possibly serves as an indication that the probably infected lions might be experiencing a state of insulin resistance. This, however, remains uncertain due to possible limitations of the assays used in this study (canine cytokine and human insulin assays) and the possible effects of the anaesthetic drugs on insulin (see Chapter 4 section 4.4.2). Additionally, when the lions were divided into the *M. bovis* exposure groupings insulin did not correlate significantly with CRP in either the exposed or unexposed populations. This limits the assumption of impaired insulin functioning to only the probably infected subset lions but not the *M. bovis* exposed lion group as a whole.

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Similar correlations were observed after the lions with cytokine outlier values and the one lion with the outlier insulin concentration were excluded from the analysis. Exclusion of the above mentioned outliers did result in the quantitative QFT GEA values having a Spearman's correlation coefficient of more than 0.5 with cortisol (Figure 6.2). This correlation was highly significant ($p < 0.0001$). Dividing the lions into the exposure groups showed that the QFT GEA values correlated significantly (Spearman's $r = 0.57$, $p = 0.0046$) with cortisol concentrations in the exposed lions but this was not the case for the unexposed lions. As mentioned in Chapter 2, the QFT GEA measures MIG gene transcription in response to IFN-γ production in blood samples stimulated with *Mycobacterium tuberculosis* complex antigens and does not measure IFN-γ concentrations directly. Cortisol has been shown to have immunosuppressive and/or anti-inflammatory functions (Mahuad et al. 2004; Rook, 1999) with one of these functions being the suppression of IFN-γ production (Brand et al. 2001; Mahuad et al. 2004; Petrovsky et al. 1998). However, there are some indications that these actions might differ between species, type of disease, and at different sites of action (i.e. tissue types) (Fisher et al. 1997; Nogueira & Silva, 1997; Rook, 1999). Since the QFT GEA does not measure IFN-γ directly, one should not expect to see an inverse correlation of cortisol with the GEA as was previously described for cortisol and IFN-γ (Brand et al. 2001; Petrovsky et al. 1998). The current positive correlation between cortisol and the QFT GEA should rather be interpreted as follows: Since the stress of a chronic disease/infection such as caused by *M. bovis* has the ability to activate the hypothalamic-pituitary-adrenal (HPA) axis and thereby stimulate cortisol production (Rook, 1999; Yildiz et al. 2005) and since the presence of an *M. bovis* infection would lead to increased IFN-γ gene transcription and thereby MIG production under antigen stimulation it can be expected that an increased cortisol concentrations would be indirectly associated with increased IFN-γ gene transcription in antigen stimulated blood samples from lions exposed to or infected by *M. bovis.* Since the actual IFN-γ levels were not measured it is, however, not possible in the current study to infer possible immunosuppressive actions of cortisol on IFN-γ production. Additionally, as was shown in Chapter 3, the possibility exists that factors other than *M. bovis* might be driving the observed differences of cortisol concentrations between wild and captive lions, thereby limiting the ability to make definitive conclusions.

6.4.2 Cross systems investigation for lions classified as probably infected and uninfected

While the above gives some insights into differences observed between the *M. bovis* exposed and unexposed lions, more insights might be gained by looking at the lions suspected of having a high probability of being infected with *M. bovis* and those that are probably uninfected[6]. As mentioned before, lions with outlier cytokine values were not excluded from analyses done with the data from the probably infected and uninfected lions, however, the one lion with an outlier insulin concentration was excluded. Exclusion of this lion led to a slightly better differentiation

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[6] The probably uninfected lions include lions from both the exposed and unexposed populations. See Chapter 2, section 2.5 for description of selection criteria that lead to the subsets of lions classified as probably infected or probably uninfected.

between probably infected and uninfected lions on the PCA score and correlation plots (Figure 6.7 vs. Figure 6.8). Additionally it resulted in more biological markers contributing to the 1st principle component (Table 6.2) and an increase in the Spearman's correlation coefficients for the biological markers that had Spearman's correlations of ≥ 0.5 (Figure 6.5 vs. Figure 6.6).

The majority of biological markers contributing the most to PC1-2 came from the energy metabolism biomarkers (glucose, leptin, BMI) while the major contributors to PC2-2 had immune system origins (QFT GEA values and cortisol). Differentiation between the subsets of probably infected and uninfected lions on the PCA score and correlation plots (Figure 6.8) were not as clear as was seen for the exposure groupings (Figure 6.4). This could possibly be ascribed to the fact that the exposure groupings were synonymous with wild vs. captive, resulting in location biased differentiation that is lost to some degree in the probably infected cohorts since some wild lions together with captive lions were classified as probably uninfected. Differentiation between the probably infected and uninfected subsets occurred along both the PC1 and PC2 axes with probably uninfected lions having lower QFT GEA (discussed in Chapter 2) and cortisol (discussed in Chapter 3 section 3.4.1) values, and higher BMI, leptin, and glucose (discussed in Chapter 4 section 4.4) values than the probably infected lions. Note: The differentiation on the basis of QFT GEA values is to be expected since the classification of the probably infected and uninfected subsets was done taking the GEA values into account.

Inspection of the scatterplot matrix drawn with the data of the probably infected and uninfected lions (insulin outlier excluded) showed that CRP (immune and stress marker) had Spearman's correlation coefficients of > 0.5 with BMI and insulin (energy metabolism markers). Additionally, the Spearman's correlation coefficient for cortisol and QFT GEA values increased from 0.63 in the exposure grouping to 0.75 in the infection grouping.

While insulin and CRP had a positive correlation when the biological markers of the probably infected and uninfected lions were grouped together, division of the data into the respective infection classifications showed that insulin and CRP did not correlate in the probably infected lions. For the probably uninfected lions the correlation between CRP and insulin remained significant (p = 0.0443). This might limit the speculation that *M. bovis* exposed/infected lions are experiencing impaired insulin sensitivity. Rather, the fact that insulin and CRP significantly correlates in the uninfected lions but not the infected lions might indicate that the human insulin assay used was not sufficient for insulin analyses in lion samples, further limiting relevant conclusion to be drawn. However, since numbers are low, the study is probably underpowered and a lack of correlation should not be taken as definitive in this case.

The negative correlation between CRP and BMI was significant (p = 0.0013) when all the lions from the probably infected and uninfected subsets were grouped together. Separated into the respective probably infected or uninfected groupings neither the infected nor the uninfected lions showed a significant correlation between CRP and BMI, however, the general trend of increased CRP and lower BMI's remained. This is in contrast to previous findings in humans that showed a

significant positive correlation between CRP and BMI (Forouhi et al. 2001; Park et al. 2004; Rawson et al. 2003; Rexrode et al. 2003). It should be noted that the aforementioned studies were conducted on healthy individuals with no pre-existing inflammatory disease. A large part of this positive correlation in healthy humans is based in the ability of adipose tissue to produce pro-inflammatory cytokines and thereby increased production of CRP. It could therefore be speculated in the current study that the inverse correlation is a result of increased CRP due to *M. bovis* infection associating with decreased BMI scores as a result of disease. However, this does not explain why the general trend of decreased BMI with high CRP is still present in the uninfected, presumably healthy lions. A possible factor that might be in play is the type of adipose tissue present. There is some indication that visceral/abdominal adipose tissue secretes greater proportions of pro-inflammatory cytokines such as IL-6, an important regulator of CRP, than does subcutaneous adipose tissue (Park et al. 2004; Rexrode et al. 2003). Considering that BMI does not differentiate between types of adipose tissue and in humans, differences in the relationship between CRP and adiposity has been reported in different ethnic groups (Forouhi et al. 2001), it might be that in lions the major adipose tissue contributing to BMI is not of the type that contributes to pro-inflammatory cytokine production, possibly limiting the contribution of adipose based CRP to total circulating CRP. This will need further investigation.

The correlation between cortisol and the QFT GEA results were highly significant ($p < 0.0001$), however, when the lions were split up into the probably infected and uninfected subset groups only the infected group showed a significant correlation ($p = 0.0485$) between cortisol and QFT GEA values. This is similar to what was described above when *M. bovis* exposed and unexposed lions were compared (see section 6.4.1). The reason for the more significant correlation in the exposed lions compared to the probably infected lions is unclear but could possibly be ascribed to the much smaller sample size of the probably infected lions.

To see if a combination of immune/inflammatory and energy metabolism markers could help to distinguish between *M. bovis* infected and uninfected lions, a cluster analysis was done and a dendrogram drawn (Figure 6.9). Using all of the markers, no clear distinction between probably infected and uninfected lions could be observed. Because of this and because some overlap between the probably infected and uninfected lions were also present in the PCA (Figure 6.8), possible differentiation between the two subsets of probably infected and uninfected lions by decreasing the markers used in the analyses and only selecting markers that contributed the most to PC1-2 (Table 6.2) and markers that had Spearman's correlation coefficients of ≥ 0.5 with at least one other marker (Figure 6.6) was done. This reduced the number of markers used in the analyses from 14 to nine. However, with this reduced number of markers, a cluster analyses and dendrogram (Figure 6.10) still did not show a clear differentiation between probably infected and uninfected lions. This lack of differentiation can probably be explained by the fact that many of the markers used are not solely affected by the presence or absence of *M. bovis* and are likely influenced by various other factors not controlled for in this study. This might be especially true for

the energy metabolism markers whereby the type of anaesthetic drug used, the type of diet, and the time between meals might all have effects on the markers measured.

6.4.3 Cross systems investigation of the male lions' biological markers

In the current study the only reproductive endocrine hormone that could be measured was testosterone (See Chapter 5 for discussion on other hormones and hormone assays). Therefore a cross system investigation with reproductive hormones included was done for only male lions. It should be noted that during the course of this study the initial testosterone assay (Siemens RIA) was discontinued. This resulted in all of the wild lions assayed with one assay (Siemens RIA) while all, except one, captive males were assayed with another assay (IBL RIA). Therefore, for the statistical analyses in this chapter, wild lion testosterone results obtained with the Siemens assay adjusted to the levels of the IBL assay were used (See Chapter 5 section 5.2.3.1 for more detail on this).

From the results discussed in Chapter 5, it was established that age related differences also occur between male lions' testosterone concentrations. Dividing the male lions into the different exposure and/or infection groupings while accounting for age, in some instances resulted in small sample sizes that were not appropriate to proper statistical analyses. Therefore, in the initial investigation (i.e. Scatterplot matrix and PCA) the data set was not divided up according to age classes. When age class related investigations were done, the KNP age classification system was used. This was done because the KNP aging system compared to the Brown aging system gave testosterone results more in line with results described in previous studies (See Chapter 5 section 5.4.1). Additionally, for the purpose of maintaining a greater sample size, lions with outlier cytokine values were not excluded from the analyses.

The PCA of all the markers, including testosterone, showed that for male lions the biological markers that contributed the most to PC1 were insulin, BMI, QFT GEA, and CRP. The categorical variables of age class, *M. bovis* exposure, and *M. bovis* infection were super imposed upon the PCA score and correlation plots. Differentiation between the exposure groupings (Figure 6.13) and between the infection groups (Figure 6.14) were relatively clear while much more overlap was seen for the different age classifications (Figure 6.12). From the PCA score and correlation plot (Figure 6.12) it would seem, as could be expected, that adult males associated more with higher testosterone concentrations and greater BMI scores than did the juvenile males. The juvenile males showed a greater association with increased CRP, cortisol, and insulin. The sub-adult males overlapped more with the adult males than with the juvenile males. Differentiation between the exposed and unexposed male lions (Figure 6.13) occurred in a similar fashion to what was seen between adult and juvenile males, with unexposed males associating more with increased BMI scores and testosterone concentrations and the exposed males associating more with increased CRP, insulin, and cortisol. These findings are not necessarily surprising and might be simply an age effect. The lack of sufficient animals to explore this properly could introduce

significant bias to the study. This apparent similarity can to a large part be explained by the fact that all juvenile males in the current study originated from the exposed lion population. Closer inspection of the score and correlation plots when comparing Figures 6.12 and 6.13 with each other show that the two adult males responsible for the overlap with the juvenile males originate from the *M. bovis* exposed population. Interestingly these two males also had low BMI scores and low testosterone concentrations with one of those males also grouped with the probably infected lions (Figure 6.14). With regards to the probably infected and uninfected subsets, uninfected males, while having higher BMI scores and testosterone concentrations, associated more with increased glucose and leptin values. The probably infected males had a closer association with increased CRP and insulin values. When one considers all three of the plots discussed above, it might be easy to assume that the association of the unexposed and uninfected lions with higher BMI scores and increased testosterone is due to sampling bias with more adult males sampled in the unexposed and uninfected populations. However, as was shown, closer inspection revealed this not to be the case when the two adult males with lower BMI scores and testosterone concentrations were also grouped with the exposed and infected population lions.

On the PCA score and correlation plot, the correlation lines of BMI and testosterone are almost identical in length and direction indicating a possible strong positive association between these two markers. This possible association was confirmed when a scatterplot matrix of all the male markers was compiled (Figure 6.11). Testosterone and BMI had the greatest Spearman's correlation coefficient (Spearman's r = 0.86, p < 0.0001) of all the biological markers measured in the male lions. Interestingly, when the males are divided up into the exposure groupings, BMI and testosterone only correlates (Spearman's r = 0.85, p = 0.0001) in the wild/*M. bovis* exposed males. Division into the probable infection groupings showed that for both the probably infected and uninfected males BMI and testosterone have exactly the same correlation (Spearman's r = 0.94, p = 0.0167). Without accounting for the exposure or the infection groupings BMI and testosterone only correlated in the juvenile males (Spearman's r = 0.83, p = 0.0047). It is unclear why the rest of the age groupings or the unexposed lions did not show a correlation between BMI and testosterone since one would expect that as males mature and grow that the BMI and testosterone would increase. It could be speculated that the lack of correlation for the unexposed/captive males are due to the significantly (MW test, p = 0.0004) greater BMIs when compared to the wild/exposed lions, possibly indicating that they were "fat". However, since the majority of captive males were fully grown while the majority of wild males were not, it might be speculated that the social hierarchy of captive males are contributing to lower testosterone concentrations in less dominant mature males, thereby lessening the correlation between BMI and testosterone as males mature and social dynamics starts to play more of a role. Another possibility might be that the Siemens testosterone assay gives better representative testosterone results than the IBL assay. This, when one considers that all of the exposed males were assayed with the Siemens assay and all but one of the unexposed males were assayed with the IBL assay; that three of the six probably uninfected

lions were assayed with the Siemens assay; that the majority of the adult and sub-adult males were assayed with the IBL assay; and that only the sub groupings containing a good representation of lions assayed with the Siemens RIA showed positive correlations between BMI and testosterone. Because the testosterone concentrations of all the juvenile lions were adjusted to IBL levels it was possible to double check if the correlation between BMI and testosterone using the original Siemens results would differ from that obtained with the adjusted testosterone values. With the Siemens testosterone values juvenile BMI and testosterone showed a stronger correlation than when concentrations adjusted to IBL values were used. Therefore, the lack of correlation between BMI and testosterone for the unexposed males, the adult males, and the sub-adult males could possibly be an artefact from the assay used.

If it is the case that the Siemens RIA gives a better representation of total testosterone than does the IBL RIA, more value is given to the observation that two of the three *M. bovis* exposed adult males (all assayed with Siemens RIA) are grouped in the vicinity of the infected males on the PCA score and correlation plots while the third adult male is grouped with the probably uninfected lions. Although speculative, since a variety of other factors could affect BMI, this might serve as an indication that reduced BMI due to *M. bovis* disease is accompanied with reduced testosterone production. The reduction in testosterone might have negative implications in a male lion's ability to compete in lion society, as was seen to a degree in the captive male lion discussed in Chapter 5 section 5.4.1.2. Additionally, in human and other non-human animal models high testosterone levels together with low cortisol levels is believed to promote social aggressive behaviour (Montoya et al. 2012). Inversely, high cortisol levels together with low testosterone levels are believed to facilitate social withdrawal behaviour (Montoya et al. 2012). Furthermore, as reviewed by Viau (2002) there is evidence that both these hormones show reciprocal interactions with high concentrations of one inhibiting the production of the other. Whether the testosterone and cortisol ratio would have an effect on the social dynamics of male lions remains to be established. In the current study, cortisol and testosterone for all the male lions had a Spearman's correlation coefficient of < 0.5, however, this was still a significant inverse correlation ($p = 0.0294$). Although the correlation was not significant when looking at only the adult males, the general trend of decreased testosterone together with increased cortisol could still be observed. Considering the relatively small sample size of wild adult males in the current study, it might be worthwhile to further investigate whether the testosterone and cortisol ratio plays a part in the social behaviour of male lions since there is an indication, although not conclusively proven, that *M. bovis* infection/disease might be facilitating lower testosterone concentrations together with higher cortisol concentrations. The alternative is also possible, whereby the overall inverse correlation of testosterone and cortisol is an artefact of the age dependant differences observed for both these markers whereby juvenile lions had lower testosterone and higher cortisol concentrations than adult lions, and the majority of wild males were juveniles.

The scatterplot matrix of the male biological markers also showed relative strong inverse Spearman's correlations between testosterone and CRP (Spearman's r = 0.65, p = 0.0004) and testosterone and insulin (Spearman's r = 0.60, p = 0.0014). Interestingly neither testosterone and CRP nor testosterone and insulin showed any correlation when the males were subdivided into the exposure, infection, or age class groupings. Previously, associations of low testosterone and high CRP levels with altered energy metabolism such as in type two diabetes and disease states such as cancer has been reported in humans (Bhatia et al. 2006; Burney et al. 2012; Tong et al. 2005). Additionally, low testosterone levels have also been suggested as a risk factor for the development of the metabolic syndrome (Saad, 2009) which together with type two diabetes are the most common clinical syndromes associated with insulin resistance (Wilcox, 2005). Lower testosterone levels have also been reported in the presence of increased insulin concentrations during states of insulin resistance (Pitteloud et al. 2005). These associations of low testosterone and high CRP with states of insulin resistance, the fact that insulin production can be increased as a result of insulin resistance (Wilcox, 2005), the suggested link between TB and type two diabetes (see Chapter 4 section 4.1), and the correlations in the current study between testosterone, CRP, and insulin, together tempt speculation that lions in the current study are experiencing an altered state of energy metabolism. This might be due to *M. bovis* infection, although other factors or diseases not controlled for in the study might be at play.

Similar to what was seen and discussed earlier for the analyses with all the lions (section 6.4.1) and the analyses with the probably infected and uninfected lions (section 6.4.2), in the male lions insulin and CRP had a significant positive correlation while BMI and CRP had a significant negative correlation. Using only the male lions a significant positive correlation between insulin and cortisol was observed. Because of this and despite a Spearman's correlation coefficient of below 0.5 (Figure 6.2) the correlation between cortisol and insulin was also investigated using the data from all the lions and a significant positive correlation was observed. This is to some extent what could be expected if one considers the increased cortisol production in the presence of *M. bovis* infections (Bini et al. 2015; Bottasso et al. 2010; Bottasso et al. 2013; Bozza et al. 2007) and the assumption that in lions, TB will also be associated with a state of insulin resistance as suggested for humans. However, insulin and cortisol did not correlate significantly in the probably infected and uninfected lion subsets. This cannot be explained by the smaller sample size of the probably infected and uninfected subsets since the smaller sample size of only male lions still showed a significant correlation. Alternatively, the lack of significance in the probably infected and uninfected lion sample but not the overall sample might indicate that the differences in insulin concentrations between the wild/*M. bovis* exposed and the captive/unexposed populations are not due to TB but rather due to other unknown factors not controlled for in this study.

Many of the markers used in the current study, although having cross system interactions, are relatively specific to their metabolic systems. The melanocortin peptide α-MSH has been

shown to play a role in all three metabolic systems (Gantz & Fong, 2003) investigated in the current study. Unfortunately, attempts to analyse α-MSH in lion serum samples were not successful in the current study. Two different commercially available ELISA kits were tested. The one kit was a multi species ELISA (DRG® Alpha-MSH (Human, rat, mouse) EIA kit, EIA-2951, DRG International Inc, USA) and the other a cat ELISA(Cat Alpha-Melanocyte Stimulating Hormone (AMSH)ELISA kit, MBS078063, MyBioSource Inc., San Diego, California, USA). The melanocortin peptides all originate from a single pro-hormone (pro-opiomelanocortin, POMC). While it was not possible to get the protein sequence data for α-MSH, a search of the Ensemble data base (Ensemble, 2016) provided the POMC protein transcript sequences for the mouse (Transcript: Pomc-201 ENSMUST00000020990), human(Transcript: POMC-201 ENST00000380794), dog (Transcript: POMC-201 ENSCAFT00000006656), and cat (Transcript: POMC-201 ENSFCAT00000003658) genes. Multiple sequence alignment analyses with Clustal Omega (EMBL-EBI, 2016) showed that the POMC sequence between cats and humans was 83% similar while it was 92% similar between cats and dogs. Cats and mice only shared 78.8% similarity in POMC sequences. The possibility therefore exists that the peptide sequences targeted by the antibodies used in the ELISA's are not conserved between lions and the target species of the assays used. Additionally, the possibility of unknown substances in the lion serum interfering with both assays also exists. It might therefore be useful to develop an assay sensitive to α-MSH in lion samples for future investigations into metabolic states of lions affected by disease.

6.5. Conclusion

One of the aims of the multi-systems investigation in this chapter was to assess whether the biological markers used in the current study could give some insight into the general health of lions when comparing a population exposed to *M. bovis* to an unexposed population. Similarly, a finer scale investigation was done comparing presumably *M. bovis* infected lions to uninfected lions.

For the purpose of this study two basic methods of investigation were used. The first method using PCA's showed differentiation between *M. bovis* exposed/wild and unexposed/captive lions and between probably infected and uninfected lions with a variety of biomarkers. This differentiation was to a large part due to higher concentrations of certain energy metabolism markers and lower levels of immune/inflammatory markers in the presumably healthy lion population, while the inverse was seen for the presumably *M. bovis* exposed or infected lions.

The second method using scatterplot matrix analyses that included linear regression and correlation analyses showed significant correlations between certain markers of the energy metabolism, immune/inflammatory, and the reproductive endocrine systems. Most of these correlations, either positive or negative, to some extent could serve as indications that an altered energy metabolism associated with an increased immune/inflammatory response is present in the

wild lions when compared to the captive lions. This is also associated with reduced testosterone levels in the wild lions. It should, however, be noted that although significant correlations were observed between various energy metabolism, immune/inflammatory response, and reproductive endocrine markers, these correlations do not necessarily establish causation. While in some instances a causative link might be present, the correlations observed might only indicate that the lions are experiencing an altered metabolic state that is simultaneously affecting different metabolic systems indirectly linked with each other.

Furthermore, although results from the current study show some indication that TB is driving metabolic change in the wild lion population, a final conclusion that it is clearly the case should be cautioned. This is owing to the lack of definitive antemortem TB diagnosis in lions and the possibility of factors not controlled for in this study such as diet, other diseases, handling stress, sensitivity and specificity of assays, and effects of anaesthetic drugs possibly contributing to the observed differences. Additionally, considering that a significant proportion of wild lions were juveniles, the correlations observed in some instances might be artefacts of age related differences for some metabolic markers.

Despite this, the fact that significant correlations between markers from different metabolic systems were observed, in line with previous findings in humans during states inflammation or disease, gives some validation to the results obtained using human based assays.

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Chapter 7

Overall Conclusion

It is clear that one of the main contributing factors to the difficulty in deciding on proper disease management of TB in lions, or in fact, whether at a population level it is even necessary, is a lack of knowledge concerning *Mycobacterium bovis* infection, disease progression, and the resultant effects on lion metabolic systems and disease outcome at the scale of individuals and populations. This results in decisions pertaining to the management and importance of TB in lion populations to a large part being ignored or at best perhaps based on assumptions drawn from other animal models or from population census sampling data. In the light of the dearth of research information on TB in lions, this thesis is an attempt to initiate research into the possible effects of *M. bovis* infection and resultant disease on lions and hopefully contribute to the knowledge base of TB in lions, thereby facilitating future disease management decisions.

Relatively early in the history of lion TB studies it was established that exposure to *M. bovis* can lead to active disease and eventually death of lions. The majority of reports on TB in lions report descriptions of disease pathology. However, not much has been published on metabolic alterations associated with *M. bovis* infections or disease in lions. Simple observation of emaciation in lions with TB allows one to speculate that significant metabolic changes must be taking place during the disease process. Such changes presumably alter lion behaviour with unknown consequences for pride dynamics and ecosystem effects. Conclusions made using symptomatic and mortality data only may not provide the complete picture as it is developing, since this approach runs the risk of focussing on the effects on populations at a later stage of the disease process only. On the other hand, investigations of the metabolic systems might give insights into the effects during the sub-clinical stages of disease, which may be present in individuals for years before ultimate death. The current study therefore planned to investigate three different metabolic systems that might be affected by TB and might eventually be used to gain insights into possible population related effects of the disease. The study aimed to do this by measuring different metabolic markers using commercially available but not lion-specific tests. Note: Initial design of this study was to serve as an add-on study aimed at adding value to a lion demographic study undertaken by SANParks (the South African National Parks board) in the Kruger National Park (KNP), South Africa. This study was originally conceived to be a cohort design, where a collaboration of researchers focussed on approximately 22 lion prides that would be captured, tested for a variety of diseases and where each animal would be held for at least 72 hours for various tests, observations and diagnostics tests. There would have been follow up work done months later on the same animals to re-evaluate lion health and numbers. This thesis work began based on the detailed and extensive planning of such a "lion survey". Unfortunately, this comprehensive lion survey was not completed, since rhinoceros poaching in the KNP escalated suddenly, making the cost, logistics, and personnel availability and safety for an extensive lion

study impossible. The work in this thesis therefore has limitations of low numbers of animals and controls that had to be sampled from a number of locations other than and including the KNP.

To facilitate a comparative study, lions were sampled from distinct populations separated geographically and presumed to be under different *M. bovis* exposure pressures, for example, captive/*M. bovis* presumed unexposed lions and wild/*M. bovis* exposed lions. A fundamental part of doing a comparative study is the correct diagnosis and differentiation between such healthy and diseased animals. For the current study, four different diagnostic tests were utilised. Independently each diagnostic test gave varying results regarding prevalence of TB in both the captive and wild populations. This was not surprising since the sensitivity and/or specificity of the tests were either relatively low or not established for lions. Interestingly, and unexpectedly the diagnostic tests indicated a relatively high prevalence of TB exposure in the captive lion population. This could indicate that the captive population is also under unanticipated exposure pressure of *M. bovis*. Alternatively, a more likely alternative is that captive lions are more often exposed to mycobacterium complex organisms other than *M. bovis* (e.g. *M. tuberculosis*) due to their frequent close proximity to humans. Therefore, while the specificity of the diagnostic tests might be suitable to identify *M. bovis* infected lions in the wild, they are not necessarily suitable for diagnosis of TB, due to *M. bovis*, in captive lions per se. Rather, use of the currently available diagnostic tests in captive lions should incorporate multiple tests while the life history of the individual or group of animals tested must be considered. An additional confounder that resulted from not being able to definitively diagnose TB in lions, is the inability to accurately classify a live lion as being healthy, infected, or diseased, thereby limiting the overall value of results gained in the current study.

Given the lack of definitive antemortem diagnosis, selection criteria were compiled in an attempt to differentiate at the very least between lions that are probably infected with *M. bovis* (even in the absence of detectable clinical disease) and lions that are probably not infected by *M. bovis* or any other mycobacterium complex organism (Chapter 2). Using such subsets of lions might allow identification of metabolic alterations more likely due to *M. bovis* rather than to other environmental factors. Unfortunately, due to the magnitude of other environmental factors (and lack of power owing to low lion numbers, as explained above) that could allow differentiation between captive and wild populations, all findings in this study are indications of directions future studies could take to refute or confirm these findings. The theoretical ideal would have been to do a comparative study on healthy lions and lions experimentally infected with *M. bovis* in a situation where the majority of possible environmental effectors could be controlled for. Unfortunately this was not possible for a variety of reasons. Alternatively, additional value could possibly be gained by comparing healthy, sub-clinically infected, and diseased free ranging lion sub-populations originating from geographically and climatically similar ecosystems.

Despite the difficulties in conclusively establishing infection/disease status in individual lions, some interesting observations could be made concerning the different lion groupings. This was discussed in detail in the respective Chapters. Briefly the main findings were: that within the

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wild/*M. bovis* exposed population there are lions probably experiencing either a Th-1 type or Th-2 type immune response and that the *M. bovis* exposed lions and the probably infected lions showed a greater inflammatory and stress response than the captive lions. This stress response could possibly be ascribed to *M. bovis*, although it is acknowledged that this must be regarded as unproven and speculative at the moment (Chapter 3). There are indications that the presence of *M. bovis* cause alterations to lion energy metabolism but a variety of environmental factors could account for the differences observed, while unavoidable inconsistencies in the sampling protocols used at the different locations could also have played a role (Chapter 4). Comparatively lower testosterone concentrations within the different age classes were observed in the *M. bovis* exposed and probably infected male lions compared to the captive/unexposed or uninfected lions (Chapters 5 and 6). It was also established that a provocative test of the kisspeptin system could be used to describe lion reproductive endocrine function (Chapter 5). Note: The provocative kisspeptin challenge was only conducted on captive lions. Still, one diseased captive adult male showed reduced basal testosterone concentrations compared to other adult males and a greater proportional response to kisspeptin stimulation. This was in line with the proposed hypothesis that external factors such as disease could inhibit reproductive ability leading to lower basal testosterone concentrations that further leads to a proportionally greater response to stimulation when compared to other healthy males at the same stage of sexual development. Therefore, a provocative kisspeptin challenge would be suitable to investigate possible effects of *M. bovis* infection/disease on lion reproductive endocrinology.

The inability to conclusively diagnose lions as infected or diseased, the lack of sensitivity of some commercially available assays to measure certain biological markers in lion samples, relatively small sample sizes when age and gender were accounted for, and the possible effects of certain anaesthetic drugs on circulating concentrations of certain biological markers, are major limitations to this work and allow mostly for speculation and guidance for future work. Nevertheless, the multiple systems investigation done in the previous chapter (Chapter 6) showed associations between biological markers from the different metabolic systems that are in line with what could be expected in an infected and/or diseased state, possibly serving to increase the relevance of the metabolic differences observed between the different exposure and infection sub-groups.

At the beginning of this thesis it was stated that this study would not follow a standard format of hypothesis testing but rather that the aim would be to generate data that will contribute to the formulation of directed hypotheses for further study and that this would be guided by a set of additional aims and objectives. Conclusions drawn with respect to each of these objectives in the light of suggesting further study together with subsequent hypotheses are presented below:

<u>1) Is it possible to make use of easily obtainable, commercially available assays or laboratories, not specifically designated for analyses of lion samples, to generate usable data for the biological markers of the immune/inflammatory response, energy metabolism, and reproductive endocrinology?</u>

The current study showed that for certain biological markers it is possible to make use of commercially available human or canine assays to obtain usable data, although in some instances concentration values of biomarkers are representative rather than actual. More specifically, the current study, while making use of commercially available assays not specifically developed for lion samples, obtained usable data for lion CRP, cortisol, and testosterone concentrations. To a lesser degree, usable data were obtained for some cytokines, progesterone, glucose, insulin, leptin, and ghrelin. However, a variety of environmental factors and possible irregularities in the findings could result in the usability of these data being questioned. No usable data could be generated with human or canine assays for the biological markers, LH, oestradiol, α-MSH, %HbA1c, and various cytokines. This unfortunately serves as an indication that future studies investigating aspects of lion energy metabolism and reproductive endocrinology (more specifically of female lions) will need to use and/or develop bio-marker assays aimed at lion samples. Alternatively, investigation into the use of samples where the intended analyte has been extracted and therefore does not contain other constituents that could interfere with the assay might yet prove to be sufficient for analyses with available human/canine assays.

<u>2) Will it be possible with the data generated to distinguish between lions of different exposure or presumed infection status?</u>

Although the short answer is "yes", it unfortunately is not that simple. While this study did manage to distinguish between lions of different exposure and/or presumed infection status, there were many other factors such as diet, other diseases, handling stress, sensitivity and specificity of assays, and effects of anaesthetic drugs that could have contributed to the observed differences. Therefore, a main finding of the current study, as mentioned before, is the need for a controlled *M. bovis* infection experiment in which many environmental factors could be controlled for.

<u>3) Will it be possible with the current study model to generate relevant knowledge to describe *M. bovis* infection or disease effects on the different lion metabolic systems studied?</u>

As mentioned before some interesting observations could be made concerning the different lion groupings in the current study. Unfortunately, the lack of definitive diagnostics together with the relative small sample numbers, lack of suitable/sensitive assays, the likely role of a magnitude of environmental factors, and differences in capture protocols at the different locations all contributed to the inability to make definitive conclusions about the effects of *M. bovis* infection and/or disease on the different metabolic systems of lions. However, with this study model, it was possible to propose hypotheses (listed further on) for future investigations.

<u>4) Identify possible relationships between the different biological markers that could potentially indicate unexpected interactions, confirm usability of the assays, and/or give new insights into the possible effects of *M. bovis* in lions that could aid the direction of future research.</u>

A variety of relationships were observed between various biological markers both within and between the studied metabolic systems and were discussed under the representative chapters. In some instances the associations were in line with what could be expected in a *M. bovis* infection or diseased state while other associations did not conform to the expected. These observed associations in some instances suggested validity of the data generated by the assays used while in other instances called the validity of the data in to question. Observations that can serve as guidance for future research or directed hypotheses are included under the next point.

<u>5) Hypotheses and suggestions for future studies.</u>

Despite the variety of confounders experienced in the current study and considering the dearth of knowledge about TB in lions, it might be worthwhile proposing some hypotheses and suggestions for further study. These are by no means exhaustive, while much of the hypotheses could be considered highly speculative. (Note: Some of these suggestions and hypotheses are reiterations of those proposed in the discussions and conclusion sections throughout this thesis)

- *M. bovis* diagnostics in live lions, more frequently exposed to MTC species other than *M. bovis,* are lacking in specificity and need more validation. Alternatively, more value might be gained from developing a diagnostic test that utilises *M. bovis* specific antigens and thereby standardising the diagnosis of *M. bovis*, regardless of MTC exposure.
- In line with the above point, the interpretation of the ITT diagnostic parameters in lions frequently exposed to MTC species other than *M. bovis* (for example captive lions) will need to be re-assessed in order to reduce the prevalence of false positive results.
- Hypothesis – "In the absence of post-mortem diagnostics it is possible to use multiple antemortem diagnostic tests in concert to classify a subset of sampled lions into at least two categories of suspected infection status".
- In the light of increased cortisol associated with advanced disease in human TB patients and since, in the current study, ITT positive wild lions had higher cortisol concentrations than ITT negative wild lions, further investigations into cortisol as a possible marker of chronic disease stress in *M. bovis* infected lions will be needed.
- Due to the magnitude of environmental factors that could possibly affect cortisol concentrations measured in single time point samples, it might be worthwhile to investigate the use of hair samples to better describe cortisol concentrations of individual lions over a prolonged time period.
- Hypothesis – "Since cortisol responses are influenced by a variety of factors other than disease it might be that increased cortisol has an inhibitory effect on the hosts' antimicrobial

abilities. Therefore, individual lions with innate higher circulating cortisol concentrations are at a greater risk of *M. bovis* infection and subsequent disease".

- In the light of studies on other animal species together with the findings of the current study (using a canine based assay), further investigations into the measuring and use of cytokines in lion TB studies should be considered as it could give valuable insights into *M. bovis* disease processes and pathologies while also having the ability to contribute to infection and or disease diagnosis. (See Chapter 3 section 3.5 for more specific suggestions for further cytokine studies)

- Hypothesis – "Cytokine IP-10 is a sensitive diagnostic tool for discerning between *M. bovis* infected and uninfected lions".

- The current study was unable to conclusively link observed differences in biological markers of energy metabolism to *M. bovis* infection or disease. This was to a large part due to the inability to control for a variety of environmental and anthropogenic factors that could affect these markers within a relatively short time period together with a lack of suitable assays. Consideration of these factors (discussed in Chapter 4) will be needed in future studies before final conclusions can be drawn on the effect, or lack thereof, of *M. bovis* on lion energy metabolism/homeostasis.

- The findings obtained during the provocative Kp-10 stimulation test of the HPG axis in captive male lions in the current study confirms the usability of such a provocative test to investigate the hypothesis that "TB will compromise reproductive capacity even if it does not cause major morbidity or mortality. Owing to the sensitivity of KP neurons to alterations in metabolism, this will translate into decreased GnRH production and ultimately decreased reproductive hormones". Development of an assay sensitive to lion LH will give additional value to such an investigation by not limiting the study to only male lions but also making it possible to include females in the investigation.

- Hypothesis – "The up-regulated inflammatory response to TB is contributing to a state of insulin-resistance in diseased lions".

- Many of the above mentioned suggestions and/or hypotheses call for a controlled experimental infection study in order to reduce the magnitude of effects from environmental factors that could not be controlled for in the current study.

Chapter 1 included a review of what is known on TB due to *M. bovis* in lions and a variety of areas that were in need of information to aid in the decisions made by disease and conservation managers were identified. In an attempt to further place the findings of the current study, not specifically addressed above, in the context of the field of lion TB research I will attempt to address this in the form of questions and answers while in some instance also proposing some additional hypotheses for future studies.

1. Which metabolic system should be targeted for analyses? – Looking at the different metabolic systems independently it seems as though immune/inflammatory markers are better candidates for distinguishing between probably infected and uninfected lions. However, considering the results obtained when the data from all three systems were combined, then it would be better to target a selection of biological markers from all three systems, since this will give you a better idea of the overall health of the lion. In this regard, standardisation of the sampling procedure in terms of anaesthetic drugs used and time frame of the sampling procedure will be important to generate data comparable between sample populations.

2. Do *M. bovis* infections and/or disease have the ability to affect lion populations? – This question cannot be addressed by the data presented in this thesis. The findings of the current study indicate that the *M. bovis* exposed lions and the probably *M. bovis* infected lions might be experiencing an increased immune/inflammatory response. There is also an alteration in energy metabolism and at least for male lions, decreased testosterone production. Since chronic stress has been shown to inhibit reproductive abilities in a variety of mammals and that an energy deficit also reduces reproductive capabilities, the findings from the current study do not bode well for infected lions and perhaps lion populations. The latter point cannot be proven with the data available in the current study and together with the lack of female reproductive data it is not possible to draw definitive conclusions.

Despite this lack of information it might be worthwhile to present some hypotheses for future studies. Among the feline species lions are unique in that they are the only social species. Through evolution, this social nature has resulted in a variety of social behaviours and events that, amongst others, prevents inbreeding and facilitates the survival of future generations. Therefore, some of these behaviours and events will be highlighted while postulating how the findings of the current study might negatively impact lion populations. As mentioned before, stress may play a significant role in the ability of an organism to reproduce. It has been previously suggested that the stress experienced by female lions during a pride takeover could either be contributing to females not conceiving or even promote natural abortions (Bertram, 1975). Without knowing how the stress experienced during a pride takeover compares to the levels described in the current study and considering that adult female lions in the current study might more frequently be *M. bovis* infected or diseased (see Chapter 2 section 2.4.6), the possibility remains that chronic stress due to *M. bovis* could result in more frequent abortions, thereby reducing the populations' fecundity. An alternative hypothesis can also be presented when one considers that under normal circumstances only 20% of oestrus periods results in cubs (Bertram, 1975) and that females often abandon cubs when only one cub is born or the littermates die before the age of three months (Rudnai, 1973). It could therefore be hypothesised that chronic stress due to TB will result in less frequent successful births or more frequent single cub births that could possibly be followed by

abandonment of that cub, negatively affecting the fecundity of the lion population or pride. The above two examples are speculative and will need further investigation.

The average tenure of a pride male or male coalition in general is enough to sire at least one generation of cubs to the age of self-sufficiency and less often two generations. Each time new males take over a pride of females they kill all sexually immature offspring of the previous male(s) and sometimes even adult females are killed in the process. It therefore stands to reason that if pride takeovers occurred in close succession to each other owing to lion mortality or displacement as a result of TB that it would result in fewer generations being born to a female pride as well as fewer lions surviving to adulthood. The presence of TB could theoretically result in a faster turnover of males by reducing the fitness of a male to compete with intruders. This could be due to reduced physical ability because of the morbidity associated with TB or alternatively the reduced testosterone to cortisol ratio (see Chapter 6 section 6.4.3) might influence a diseased male's willingness to fight off competitors. Indeed previous reports on *M. bovis* infections in lions from the KNP reported situations of faster territorial male coalition turnover with consequent infanticide, and the eviction of entire male and female prides from territories (Keet et al. 2009; Michel et al. 2006). However, this topic can be still debated. The study by Ferreira and Funston (2010) reported no discernible negative effects on lion populations in areas where disease prevalence in prey animals are high. More recently, the study by Kosmala et al. (2016) while modelling TB disease dynamics for KNP lions, reported a possible ~3% population decline in KNP lions over the next 50 years due to *M. bovis* after which the population would stabilise at the reduced level. They did mention that this prediction was based on the assumption of no additional aggravating factors and inclusion of many assumptions in the model (Kosmala et al. 2016).

If indeed it is true that currently the lion population is not affected negatively by *M. bovis,* the question of how much stress a population can manage before it manifests in major change still remains. At the moment, and from the results in this thesis, it seems as though the lion population sampled in the KNP is under a higher baseline stress burden when compared to captive lions. There is some indication, although not conclusive, that this might be due to *M. bovis*. Uncertainty remains as to how the lion population will be affected should additional stressors such as from other diseases or during times of food shortages be present.

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